TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Allgemeine Lebensmitteltechnologie

DNA-based analytical strategies for the detection of genetically modified organisms and of allergens in foods

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. W. Schwab
Prüfer der Dissertation:	1. Univ.-Prof. Dr. K.-H. Engel
	2. Priv.-Doz. Dr. L. M. Niessen

Die Dissertation wurde am 16.03.2009 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 08.07.2009 angenommen.

First and foremost I would like to thank Prof. Dr. Karl-Heinz Engel, my doctoral advisor, for the opportunity to work on this challenging and very interesting topic at the Chair of General Food Technology of the Technical University of Munich. His scientific expertise, guidance and constructive critic always supported me.

I would like to thank Prof. Dr. Schwab and PD Dr. Niessen for participating in the dissertation committee.

I also gratefully acknowledge Dr. Ulrich Busch at the Bavarian Health and Food Safety Authority for providing the technical facilities for the experimental work and his awesome support that helped me during my research. A special thanks to Dr. Christine Hupfer, Clarissa Günster, Michaela Bunge, Beate Mühlbauer, Krimhilde Posthoff, Roswitha Dorfner and the whole team.

Thanks to all my colleagues at the Chair of General Food Technology, in particular Richard Röhlig, Márti Dregus, Anja Demmel, Andreas Miller, Francisco Moreano, Tobias Müller, Hedwig Strohalm, Iulia Poplacean, Ludwig Ziegler, Thomas Frank, Ruzha Babekova, Gabriele Taubert and Gerda Röske for their daily support and friendship that helped me through the years.

I would like to thank my parents, my brothers Robert and Martin, and my friends for their love, support and understanding during the years of my education.

Finally, a very special thank you to my husband who always inspired me to complete my work.

In remembrance of

Bertrand Seumo Meuleye

"Zwei Dinge sind zu unserer Arbeit nötig: Unermüdliche Ausdauer und die Bereitschaft, etwas, in das man viel Zeit und Arbeit gesteckt hat, wieder wegzuwerfen."

Albert Einstein

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List of Abbreviations

A	Adenine
bp	Base pair
C	Cytosine
CaMV	Cauliflower mosaic virus
C_T	Threshold cycle
CGE	Capillary gel electrophoresis
CRM	Certified reference material
CTAB	Cetyl trimethylammonium bromide/ hexadecyltrimethylammonium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
ds	double stranded
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FAM	Fluorescein
FRET	Fluorescence resonance energy transfer
G	Guanine
GMO	Genetically modified organism
LIF	Laser-induced fluorescence
LOD	Limit of detection
LOQ	Limit of quantification
LPA	Ligation-dependent probe amplification
nt	Nucleotide
P-35S	35S Promotor of the Cauliflower mosaic virus
PBS	Primer binding site
PCR	Polymerase chain reaction
PNA	Peptid nucleic acid
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
T	Thymine
TAMRA	Tetramethylrhodamine
t-NOS	Nopaline synthase terminator of *Agrobacterium tumefaciens*
TRIS	Tri(hydroxymethyl)aminomethane
UNG	Uracil-N-Glycosylase

1 Introduction and objectives

Stringent and transparent regulatory frameworks regarding the use of genetically modified organisms (GMO) and GMO-derived material for the production of foods and feeds have been enforced by the recently established Regulations (EC) 1829/2003 and (EC) 1830/2003 [1, 2]. Genetically modified foods have been removed from the scope of the so-called Novel Foods Regulation and its amendments [3-6]. Harmonized provisions for the risk assessment and authorization of GMO, as well as traceability, labeling and post-marketing surveillance of the use of GMO in the food and feed chain have been introduced. Labeling requirements are now independent from the detectability of recombinant DNA or proteins although thresholds for adventitious or technically unavoidable presence of GMO in food and feed have been set.

To be able to control the compliance with these legal provisions, appropriate methods for detection as well as quantification of GMO are required. The European Commission's Reference Laboratory provides official methods for the event-specific detection and quantification of material from authorized GMO. Despite these valuable methods major analytical challenges to the development and establishment of assays arise from the forthcoming authorized GMO, GMO lines close to authorization, which have benefited from a favourable risk evaluation by the European Food Safety Authority, and unauthorized GMO.

Within this context, the objective of the following study was to develop alternative strategies for the detection of GMO using screening approaches that cover different levels of specificity. Analytical challenges related to the steadily increasing number of GMO that needs to be detected should be simplified by the simultaneous detection of multiple targets using ligation-dependent probe amplification (LPA). In detail, probes for the detection of the reference genes in the genome from maize, soya and rapeseed, the CaMV 35S-promotor as screening element, the construct-specific 35S-pat junction, and the event-specific regions of the transgenic maize line MON 810 and of Roundup Ready soya were combined in a single approach.

Furthermore, the suitability of the technique for simultaneous quantification of multiple GMO lines should be assessed to offer alternative tools to real-time PCR used as default methods for GMO analysis. The possibility to quantify different transgenic maize lines in a single reaction should be analyzed detecting Bt11, Bt176, and MON810 by LPA. Calibration curves by analysis of certified reference materials were generated to evaluate the quantitative properties of the system.

In industrialized countries 1-2 % of adults and up to 8 % of children and adolescents are affected by food allergies. The increasing occurrence of food allergies and the possible health risk of unaware consumption of allergenic ingredients yielded in improved labeling regulations in the European Union. Currently, 14 different food and food groups are affected by the legislation and listed in Annex IIIa of the Directive 2003/89/EC [7, 8]. Therefore, appropriate detection methods are needed to assure the compliance of allergen labeling by surveillance authorities and for the quality control in the food industry.

Major challenges are the needs to check for the presence of food allergens at extremely low levels and to detect trace amounts of hidden allergens in composite and processed foods [9]. Allergens are mainly proteins whose routine food analysis is based on immunological detection using specific antibodies. If the allergen itself cannot be targeted, PCR-based methods amplifying specific DNA sequences offer alternative tools [10, 11].

The aim of the studies was to develop DNA-based detection methods that cope with the variety of allergens that have to be declared with the ingredients list. The LPA technique should be used for the simultaneous detection of multiple allergens in a single reaction. Unambiguous sequences should allow specific discrimination of closely related species. Experiments to study the sensitivity and the suitability of the method to detect trace amounts of allergens in complex food matrices should be performed. Suitable reference materials had to be prepared and tested with the developed assay. Method performance criteria of the developed approach should be compared to real-time PCR and protein-based ELISA methods.

Additionally, a real-time PCR method that includes a sequence-specific hydrolysis probe for the specific detection and verification of DNA from cashew nut in food should be developed. The discrimination of closely related species as well as a high sensitivity of the method should be achieved by choosing the respective target sequences. Self-prepared reference materials and commercially available food should be used to study the application of the approach to different food matrices.

2 Background

2.1 Analysis of GMO

2.1.1 Legislation in the European Union (EU) regarding the use of GMO in the food and feed chain

Many countries have established regulatory frameworks regarding the use of recombinant DNA-techniques in the course of the production of foods and food ingredients [12]. In addition to safety aspects, labeling of foods derived from GMO is a central issue of the public debate.

In the European Union, legal requirements for the labeling of GMO-derived foods had first been provided by the so-called Novel Foods Regulation [3] and its amendments [4-6]. The need for labeling was triggered by the detection of either protein or DNA from the GMO. This initiated the first wave of analytical approaches mainly focusing on the detection of DNA from GMO via qualitative PCR. Recently, new regulations have been enforced containing harmonized provisions for the risk assessment and authorization of GMO, as well as traceability, labeling and post-marketing surveillance of the use of GMO in the food and feed chain [1, 2]. Regulation (EC) No 1830/2003 defined the establishment of a traceability system allowing the documentation and the monitoring of the flow of GMO and GMO-derived products at all stages along the food and feed chain. According to Regulation (EC) No 65/2004, information on traceability includes a unique code identifier for the respective transformation event, in the case of products consisting of or containing GMO [13]. With this novel strategy labeling requirements have been extended to highly processed products and are no longer dependent on a positive testing of recombinant DNA or protein. However, it is acknowledged that in agriculture adventitious contaminations with traces of GMO-derived material cannot be excluded. Therefore, the current regulations determine certain thresholds for the adventitious or technically unavoidable presence of GMO in food and feed. Labeling is not required if the proportion of material containing an authorized GMO is not higher than 0.9 % (considered individually for each ingredient). A limit of 0.5 % has been set for products containing material derived from non-authorized GMO, which have benefited from a favorable risk evaluation by the European Food Safety Authority. No levels are tolerated for material derived from non-authorized GMO.

To be able to control the compliance with these legal provisions appropriate methods for the detection as well as for the quantification of GMO are required. The European Commission's Reference Laboratory provides official methods for the event-specific detection and quantification of material from authorized GMO. Despite these valuable

methods major analytical challenges arise from the increasing number of authorized and unauthorized GMO, the lack of reference materials and the need to determine GMO contents in composite and processed foods.

2.1.2 Qualitative PCR

PCR permits the detection of minute amounts of specific DNA sequences. The technique enables exponential amplification of a specific DNA fragment *in vitro* using short oligonucleotides (primer) flanking the sequence of interest and a thermostable DNA polymerase. In general, one reaction cycle consists of three steps allowing melting of double-stranded DNA, annealing of the primer and enzymatic elongation. Running of multiple cycles of this exponential amplification leads to a detectable quantity of the desired DNA fragment. Detailed descriptions and applications of this widely used methodology have been published [14, 15]. Every gene of interest, e.g. a transgene, can be amplified in this way. Following the PCR, the amplicons formed are generally separated according to their lengths via gel electrophoresis. Verification of their identities is achieved by cleavage with restriction enzymes and subsequent separation of the digestion products via gel electrophoresis, by Southern blotting or by direct sequencing.

2.1.2.1 Requirements of DNA preparation

In analogy to other analytical approaches, appropriate sampling is the first requirement to be met when subjecting foods to DNA-analysis. Inhomogeneous distribution of GMO in bulk materials can be a major contributor to overall analytical variance. Errors may occur at the various stages (sampling, sub-sampling, preparation of aliquots for analytical steps). Sampling strategies are especially important if low GMO concentrations are to be analyzed [16, 17]. One of the objectives of the European Network of GMO Laboratories (ENGL) is to identify and to develop sampling strategies to support EU legislation [18].
The next essential step is the extraction of sufficient amounts of DNA from the food matrix exhibiting the quality required for successful PCR analysis. A broad spectrum of DNA extraction methods is available [17, 19-21]. In general, plant tissue is ground, a detergent is applied to disrupt the cell membranes and to inactivate endogenous nucleases, different agents are used to remove proteins (Proteinase K), polysaccharides (CTAB) and lipids (chloroform), and finally the DNA is isolated by alcohol/salt precipitation [17, 22]. Various extraction kits are available, in which the purification of DNA is achieved by chaotropic salts on silica columns or by binding the DNA on magnetic particles. At present the CTAB-method and DNA-binding silica materials are most commonly used for isolation of DNA from GMO samples.

Various components of the matrix analyzed or chemicals applied during DNA extraction may influence the purity of DNA and inhibit the PCR reaction [23]. To overcome false negative results the use of homologous or heterologous internal positive controls in the PCR amplification presents a powerful tool [24].

2.1.2.2 Specificity of PCR – Choice of target sequences

Depending on the sequences selected for PCR amplification, the detection of GMO can be categorized into four levels of specificity: screening methods, gene-specific methods, construct-specific methods and event-specific methods [16, 25]. Target sequences resulting in these different specificities of the PCR assays are schematically shown in Figure 1. Screening methods target regulatory elements commonly used in transformations, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter (P-35S) or the *Agrobacterium tumefaciens* nopaline synthase terminator (T-NOS). The detection of such sequences indicates the presence of GMO-derived DNA, but positive signals may also be due to other factors, such as naturally occurring CaMV [26]. The specificity can be increased by amplifying the sequence coding for the gene of interest. However, such gene-specific methods do not allow a distinction between different GMO carrying the same transgene. Construct-specific methods target junctions between regulatory sequences and the gene of interest. However, the complete gene construct may have been transformed into different crops. In such cases, targeting the junction at the integration site between the plant genome and the inserted DNA provides the highest level of specificity. It also allows a differentiation between authorized and non-authorized GMO containing similar transgenic constructs. In the field of GMO analysis event-specific methods have been described for the detection of several maize lines (Bt 11, Bt 176, T 25, MON 863, MON 810, NK603), canola event GT73 and Roundup Ready soya [27-42].

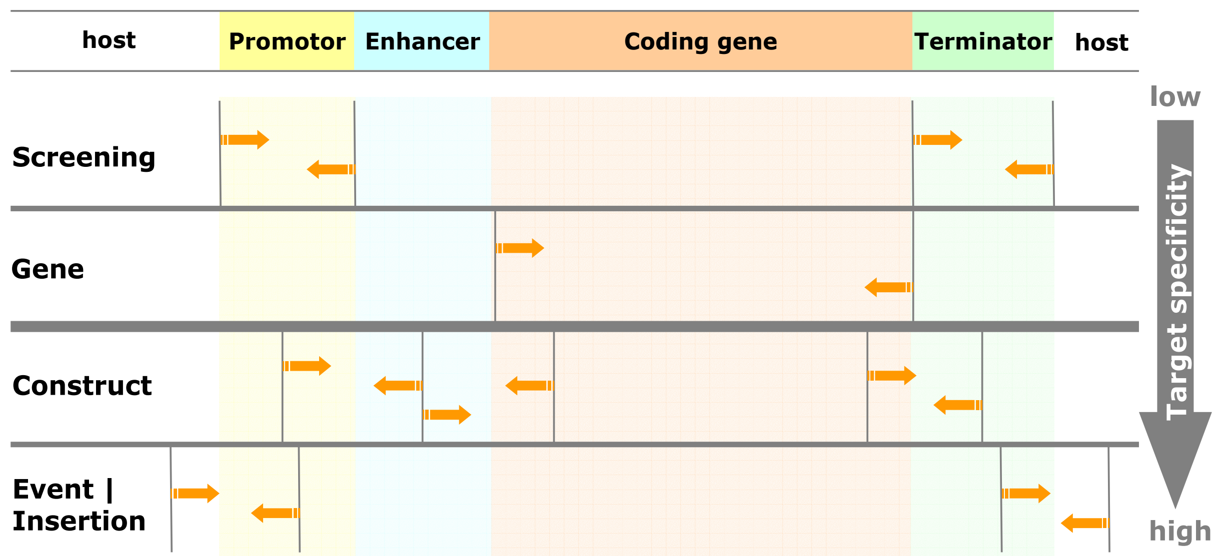

Figure 1 Target specificity of PCR assays (adapted from [25])

2.1.2.3 Influence of food composition and processing

The detection of GMO in food faces a number of challenges arising from the complexity of food compositions and the technological parameters of manufacturing processes. Food manufacturing may affect the quality and quantity of DNA in processed products. Fragmentation of DNA is initiated by shear forces, heat treatment, pH variations, enzymatic activities and fermentations resulting in reduced average size of DNA [43]. The effects of using degraded DNA as a template in PCR-based detection systems have been investigated by following diverse manufacturing practices. The choice of the size of the target sequence influences the detectability of DNA [44-46]. Using insect-resistant Bt 176 maize as example, it could be shown that the probability to detect the GMO decreased rapidly in the course of heat treatment when targeting the complete 1914 bp sequence of the synthetic *cryIA(b)*-gene. On the other hand, a shorter target sequence (211 bp), covering part of the CDPK promotor and the *cryIA(b)*-gene, was detectable even after heating for 105 min. In addition, thermal treatment in combination with acidic conditions dramatically increases the DNA degradation and thus the probability of detection [45].

Degradation of DNA in the course of food processing negatively effects the detection efficiency, especially when long sequences are the targets. This results in false negative results when analyzing samples of processed foods and feeds. Further challenges arising from the quantitative determination of GMO in composite and processed foods will be discussed in paragraph 2.1.4.

2.1.3 Quantitative PCR

2.1.3.1 Competitive PCR

Standard endpoint detection of DNA sequences as performed in qualitative PCR analysis cannot be applied to quantitative determinations due to the discontinuity of the amplification efficiency between different PCR reactions. First approaches of quantitative analysis of DNA were based on the co-amplification of the designated target and an exogenous standard (competitor) [47, 48]. In the course of this so-called competitive PCR samples containing constant amounts of template DNA are spiked with increasing amounts of the competitor. Both possess identical primer binding sites and nearly identical lengths so that equivalent amplification efficiencies in the course of the PCR reaction are to be expected. The quantification is performed by comparing the signal intensities of both amplicons, measured after gel electrophoresis. The point of equivalence, i.e. where the molar ratios of target and internal standard are equal, is determined at the intersection of the linear regression curve with the abscissa [49].

The applicability of competitive PCR for the quantification of DNA has been demonstrated for several examples [28, 50-55]. However, the labor-intensive approach requires extensive handling with PCR products and involves a high risk of cross contamination. The time-consuming and extremely material-intensive technique requires several reaction mixes for the measurement of one point of equivalence and visualization of PCR products by gel electrophoresis, in combination with complex gel documentation/evaluation.

2.1.3.2 Real-Time PCR

Real-time PCR is the state-of-the-art technique to detect and to quantify DNA. This technique permits the direct online measurement of PCR product amounts at every stage of the reaction by using fluorescence techniques. The fluorescence signals are proportional to the amounts of PCR products generated and can be observed by different approaches. As shown in Figure 2, double-stranded (ds) DNA-intercalating dyes (SYBR® green), hydrolysis probes (*Taq*Man Probes), or reversible hybridization probes (HybProbes, Beacons) are used [56-59]. The main disadvantage of double-strand-specific intercalating dyes (Figure 2A) is the unavoidable detection of non-specific PCR products such as primer dimers, besides the specific amplicons. For example, SYBR® green binds independently from the sequence to the minor groove of dsDNA. The possibility to separate the favored amplicon from unspecific background by melting curve analysis and the easy application at relatively low costs are benefits of this detection format. Another technique uses *Taq*Man probes, specific synthetic oligonucleotides hybridizing to the target DNA (Figure 2B). A probe, complementary to the target sequence, is labeled with a

reporter fluorophor at the 5'-end and with a quencher dye at the 3'-end; thus light emission is suppressed. During elongation the hybridized probe is hydrolyzed by the 5'-3' exonuclease activity of the *Taq* DNA polymerase and hence the released reporter emits fluorescence after excitation. The generated signal is proportional to the exponential amplification of templates. In the case of hybridization probes, the fluorescence resonance energy transfer (FRET) is directly measured. Two sequence-specific probes hybridizing closely adjacent to the target within a distance of one to five bases are used. As illustrated in Figure 3C, donor and acceptor probes are 3'- and 5'-terminally labeled, respectively. In case of successful hybridization of both HybProbes, the excitation of the donor is transferred to the acceptor and the emitted fluorescence can be detected. The molecular beacon, another hybridization format, is labeled on both ends with reporter and quencher fluorophor, respectively (Figure 2D). The central part of the probe is complementary to the target, whereas the terminal part is self-hybridized forming a stem-loop structure. The probe binds to the template during the annealing phase, the dyes are no longer quenched and a fluorescence signal is obtained. Advantages of internally hybridizing probes are the additional sequence-specificity and the monitoring of PCR efficiency.

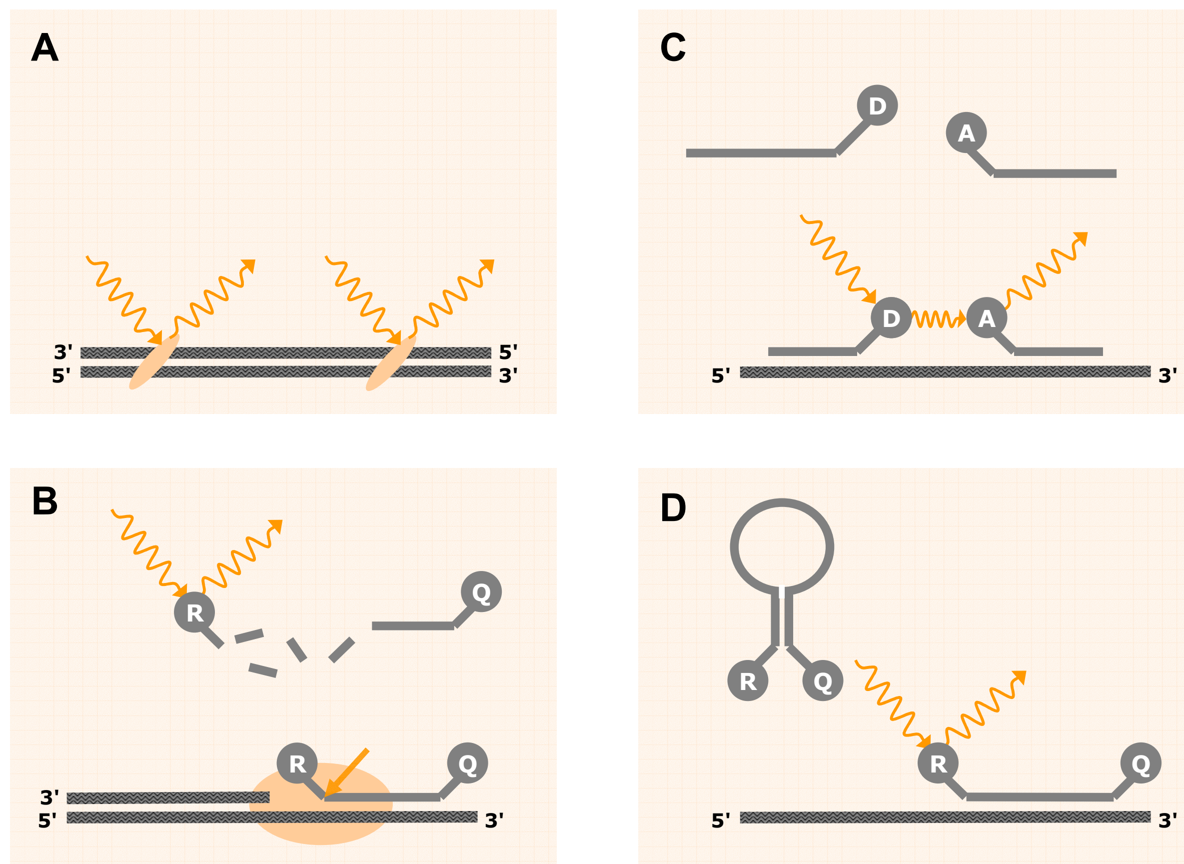

Figure 2 Commonly used detection formats of real-time PCR: (A) DNA-intercalating dye, (B) hydrolysis probes, (C) HybProbes, and (D) molecular beacon; A = acceptor, D = donor, Q = quencher, R = reporter.

At the exponential phase of the PCR amplification the template copy number of target sequences can be extrapolated on the basis of a standard curve. In Figure 3 the construction of standard curves by estimating the so-called threshold cycle (C_T) values or crossing points (C_P) from external quantification standards of known target concentration is displayed. The generated standard curve describes the logarithmic plotting of starting copy numbers and the determined C_T-values. An exact quantification of unknown samples is only assured if the amplification efficiency is equal to that of the standards used. PCR efficiency can be determined by examining the slope of the linear trend-line that should be ideally -3.32 for 100 % PCR efficiency [59].

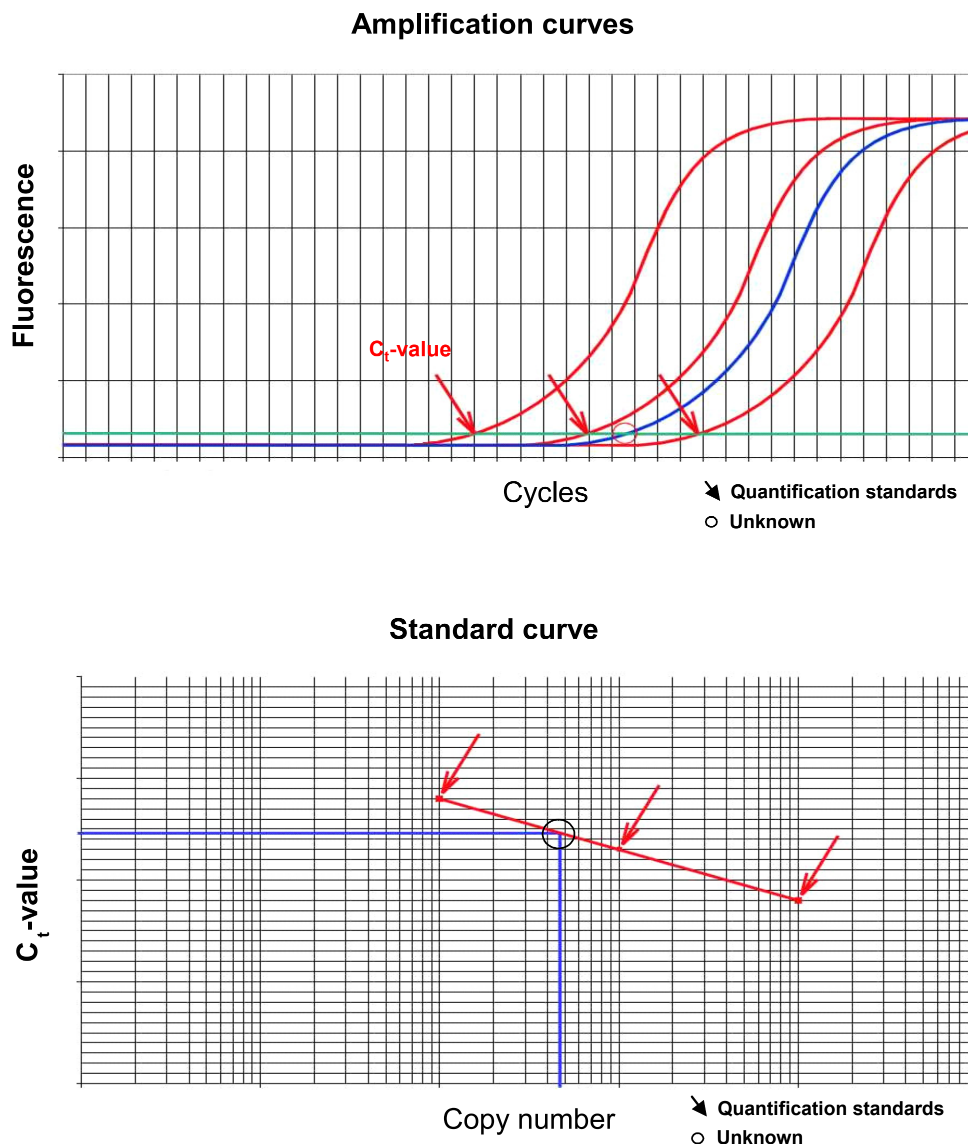

Figure 3 Principle of DNA quantification via real-time PCR. The threshold intersects the amplification curves in the exponential phase of PCR. The respective C_T-values of the quantification standards are correlated with the starting copy numbers in the standard curve.

In the field of GMO analysis the quantification in composed and processed products requires simultaneous assessment of the recombinant DNA and of a species- or taxon-specific reference gene. Thus a determination of ingredient-related GMO contents as legally required is possible. Cloned plasmid fragments [60], synthetic hybrid amplicons [61, 62] and certified reference materials (CRM standards) have been described as quantification standards [63].

The first application of real-time PCR to the quantitative analysis of GMO in foods was described by Wurz et al. [52]. The method was developed to detect a recombinant region in the genome of Roundup ready soya and a plant-specific sequence within the *lectin (le1)* gene. Special attention was paid to avoid significant differences in amplicon lengths, meeting basic requirements for its application in processed foods. The approach was afterwards tested with certified reference materials containing 0.1 % - 2 % transgenic soya, yielding results that were in good agreement with the expected data.

Approaches for the quantitative detection of Bt 176 maize and Roundup Ready soybean, targeting the transgenes *cryIA(b)* and CP4 *EPSPS*, respectively, have also been introduced [64]. The maize specific *zein (ze1)* and the soya specific *lectin (le1)* genes were used as endogenous reference targets. For the first time, PCR conditions were optimized to allow the quantification of transgenic and isogenic targets in one reaction vessel, thus eliminating tube-to-tube variations.

To date, various methods for the quantification of GMO proportions in raw food materials have been presented and validated in international interlaboratory trials [39, 40, 52, 64-69]. Commercial kits are available for the quantification of transgenic soy (Roundup Ready), maize (Maximizer™ Bt 176, Bt 11, Liberty Link™ T25, Yield Guard™ MON 810, Roundup Ready NK603 and GA21, StarLink™ and Herkulex™) and Canola (Liberty Link™). Interlaboratory testing of kits available for the quantification of Roundup Ready soybean and Bt 176 maize has been performed [69].

2.1.4 Challenges and developments

2.1.4.1 Copy number of genes – Zygosity and ploidy

The relative quantification strategy applied in GMO analysis determines the ratio of transgene copies to the respective copies of a reference gene assuming a 1:1 relationship between the two genes. In diploid homozygous lines, e.g. achieved by means of self-pollination, this prerequisite is normally met [16, 70]. However, the generation of genetically enhanced lines for commercial purposes involves cross-breeding with optimized conventional varieties. The resulting hybrids with altered levels of ploidy have lost the original correlation between transgene and reference gene. Additionally, unequal

levels of ploidy can be found in separate tissues of one organism, as for example in the diploid embryo and the triploid endosperm of maize kernels [70, 71].
The relative quantification of GMO-derived material requires special attention to be paid to the selection of suitable reference genes. Preferably, the reference target is a stable gene of known copy number in all varieties and unique to the species. A systematic evaluation of plant species-specific reference genes has shown that these criteria are not fulfilled by every candidate gene [72].

2.1.4.2 Lack of reference material – Hybrid molecules

The availability of reference materials plays an essential role in the course of the development and the validation of detection and quantitation systems of GMO as well as for the implementation of surveillance testing. Commercially available reference standards do not cover the entire spectrum of authorized GMO and there is (*per se*) a complete lack of reference material for the analysis of non-authorized GM crops.
New approaches using DNA fragments cloned in a plasmid as external calibration standards have first been described for the determination of Roundup Ready soybean [60] and are now being applied to the analysis of various GMO [29, 34, 73-74]. The synthesis of hybrid amplicon molecules by a novel two-step PCR amplification represents another strategy to obtain quantification standards. The reaction starts with separate amplification of the targeted recombinant and taxon-specific sequences with bipartite primers generating complementary overhangs. In the second PCR the mixed purified amplicons are able to self-prime due to the overhang-sequences and generate the complete hybrid molecules containing both targets [61, 62].
The application of this approach to the quantitative screening of genetically modified rapeseed lines with a number of transformation events has been described [62]. Two duplex real-time PCR assays allow the simultaneous detection of the construct-specific junction between the 35S promoter and the *pat*-gene in LibertyLink™ lines, between the *bar*-gene and the g7 terminator in SeedLink™ lines as well as the detection of a rapeseed-specific acetyl-CoA carboxylase gene. The moderate level of specificity avoids false positive results (cf. 2.1.2.2) and presents a valuable tool for the purpose of surveillance testing of GMO in food and feed products.

2.1.4.3 Quantification of DNA in composed and processed food

Industrially produced foods usually contain various ingredients. Such ingredients may be derived from the same (GM) crop but may differ significantly in techno-functional properties. For instance, mixtures of corn milling fractions with different particle sizes are

industrially applied to influence the characteristics of bakery products. It was demonstrated that unequal efficiencies in the extraction of DNA from fractions differing in particle size distributions may contribute to distortions of GMO quantification [75, 76]. For corn milling fractions a strong correlation between the degree of comminution and the DNA yields in the extracts was observed. Real-time PCR quantification of the GMO content in mixtures containing conventional and transgenic corn of different particle size distributions resulted in significant over- or underestimations of GMO contents [77].
As expected from the phenomena described for qualitative PCR, the length of the targeted DNA fragment is also crucial for the quantitative analysis of DNA in processed foods. Application of a method validated in an interlaboratory ring trial for the quantification of Bt 176 maize delivered accurate data for unprocessed reference materials but resulted in a significant underestimation of the GMO contents in heat-treated samples [69, 78]. The differences in amplicon lengths of the targeted reference gene (79bp) and the transgene (129bp) permitted the assumption that distortions in the results obtained by relative quantification resulted from the increased probability of fragmentation of the longer sequence. This could be confirmed by following the heat-induced DNA-degradation in mixtures of conventional and transgenic corn (1%) [77, 79]. Two established quantitative assays differing in the lengths of the recombinant and reference target sequences (A: Δl_A= -25 bp; B: Δl_B=+16 bp; values related to the amplicon length of the reference gene) were applied. Method A resulted in underestimated recoveries of the GMO contents in heat-treated products, reflecting the favored degradation of the longer target sequence used for the detection of the transgene. In contrast, method B resulted in increasing overestimation of the recoveries of the GMO contents in the course of the heat-treatment.

2.1.4.4 Validation

Comparable to other analytical methods, PCR-based approaches for detection and quantification of DNA also have to be validated [80]. The limits of detection (LOD) and quantification (LOQ) are method-specific but do also depend on the sample being analyzed. Three types of detection and quantification limits have been distinguished: (i) the absolute limits, i.e. the lowest number of copies required at the first PCR cycle to obtain a probability of at least 95% of detecting/quantifying correctly; (ii) the relative limits, i.e. the lowest relative percentage of GM material that can be detected/quantified under optimal conditions and (iii) the practical limits, i.e. limits considering factors such as the actual contents of the DNA sample and the absolute limits of the method [39]. Both the LOD/LOQ of the method and the practical LOD/LOQ of the test sample should be reported together with the results [39, 25].

Validation of quantitative assays for GMO in foods cannot be limited to unprocessed reference materials. Validation procedures must demonstrate that neither food composition nor processing will result in distortions of relative quantification results. Future standard protocols should target recombinant and taxon-specific sequences of nearly equal lengths.

2.1.5 Multiplex approaches

To ensure the compliance with labelling requirements appropriate analytical approaches are demanded for the detection, identification and quantification of the steadily increasing number of GMO. Multiplex assays allowing the simultaneous detection of several GMO in a single PCR reaction have been developed to meet this challenge [31, 36, 42, 81-87]. Examples of multiplex approaches for the detection of GMO are given in Table 1.
The simultaneous detection of screening elements in combination with construct-specific targets provides a profiling-like strategy for GMO analysis including unauthorized ones [81, 83, 85-86]. Other approaches partly use similar primers for the detection of different targets to overcome the difficulties of simultaneous amplification with multiple oligonucleotides in one single reaction [31, 82]. Disadvantages of these approaches using gel electrophoresis for separation are the long amplicon lengths and length differences of the targets, which pose a problem when applying the method to the analysis of processed foods [84]. At present the simultaneous amplification using nine primer pairs for the detection of different GM maize events represents the most comprehensive multiplex PCR method [87].

Table 1 Examples of multiplex approaches in GMO analysis

Targeted transgenes	Endogenous reference gene	Technique	Detection	Literature
Maize Bt11, Bt176, MON810, GA21, RR-soybean	Maize *zein*, Soybean *le1*	Multiplex conventional PCR + ligation detection reaction (LDR)	Microarray	[92-94]
NOS terminator, 35S promotor	Maize *zein*, Soybean *le1*	Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	[81]
Maize Bt11, Bt176, MON810, T25, GA21	Maize *zein*	Multiplex conventional PCR	CGE-LIF	[98]
Maize Bt11, Bt176, MON810, GA21, RR-soybean	Maize *zein*, Soybean *le1*	Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	[82]
Maize Bt11, Bt176, MON810, GA21, RR-soybean	Maize *zein*, Soybean *le1*	Multiplex conventional PCR + asymmetric PCR with labeled primer	PNA- Microarray	[88, 89]
Maize Bt11, MON810, T25, GA21		Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	[31]
P35S, NOS, *cp4-epsps*, *nptII*, *cryIA(b)*, *pat*, *bar*, *gox*, *oxy*, *barnase*	Maize *ivr*, Soy *le1*, ß-actin Canola cruciferin	Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	[83]
P35S, T35S, NOS, *cp4-epsps*, *nptII*, *cryIA(b)*, *pat*, *bar*, *gox*, Pr-act		Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	[84, 85]
Maize Bt11, Bt176, MON810, T25, GA21, NK603, MON863,TC1507	Maize *ssIIb*	Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining Capillary electrophoresis	[87]
NOS, 35S-*epsps*, *cryIA(b)*, *pat*,	Maize *zein*	Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	[86]
P35S, NOS, Amp, Maize Bt11, Bt176, MON810, T25, GA21, DBT418, CBH351	Maize *hmga*	Two-step PCR, labeled probe hybridization	Microarray	[95]
18S rRNA, NOS, NOS/*cp4-epsps*, CP4/CTP, 35S/CTP, 35S/plant, *cp4-epsps*, *nptII*, *cryIA(b)*, *bar*, PG	Maize *ivr*, Soybean *le1*, Rapeseed napin	Asymmetric PCR, labeled primer	Microarray	[42]

Additionally, qualitative applications for identification of GMO in food have been introduced using detection via microarray technology [42, 88-89]. Peptide nucleic acids (PNA) have been described as useful microarray probes for the analysis of Roundup Ready™ soybean and different maize lines [88-89]. PNA are analogues of DNA with peptides rather than pentose sugar phosphates forming the backbone. This results in high affinity of PNA oligomers to hybridize with DNA; they are more sensitive to single mismatches and thus provide higher sequence-specificity [90]. A combined assay of multiplex polymerase chain reaction and ligation detection reaction coupled with microarray has been developed [91] and applied to the analysis of traces of GMO in foods [92-94]. This approach involves an additional confirmation step of the PCR products through ligation of sequence-specific probes prior to microarray hybridization. Only the ligated products generate a fluorescence signal when hybridized to the array with their unique ZipCode sequence [91, 92]. A novel multiplex quantitative DNA array-based PCR has been presented for the quantification of transgenic maize in food and feed [95].

2.1.6 Ligation-dependent probe amplification (LPA)

One of the latest developments in multiplex approaches for quantitative analysis is the technique of ligation-dependent probe amplification (LPA). Originally, ligation-dependent PCR has been applied to the relative quantification of DNA in the field of medical diagnostics [96]. The suitability of this method for the detection and relative quantification of GMO in food samples has been demonstrated using commercially available maize standards [97]. A synthetic probe set is used for the detection of target sequences to avoid complex cloning and preparation steps required for the isolation of single stranded DNA probes. These sequence-specific probes contain a target-specific hybridization site and identical primer binding sites (PBS) at their 5'- and 3'-ends, respectively. In case of successful hybridization to adjacent sites of the target sequence, the probes are ligated by a thermostable ligase. The use of spacer sequences between hybridization sites and PBS assures ligation products with lengths characteristic for each of the target DNA. In the second step of the reaction, the ligation products are amplified competitively using one labelled pair of primers. Labelled PCR amplicons are finally separated by capillary electrophoresis and detected via laser-induced fluorescence.

The use of one pair of universal primers in the LPA method avoids one of the major difficulties of multiplex PCR applications, being the complexity of amplification reaction because of the use of multiple pairs of primers. In contrast to multiplex PCR reactions, the application of this novel approach offers great flexibility due to its modular system that can be complemented with further probes to broaden the range of target sequences. The use of synthetic oligonucleotides as probes and the employment of classical thermocycler and

detection methods enable the implementation of the technique in commonly equipped laboratories.

Detection using capillary gel electrophoresis via laser-induced fluorescence (CGE-LIF) represents a very sensitive and rapid separation and detection approach in automated manner. Different amplicons can be distinguished by size using either labelled PCR primers or DNA-intercalating dyes. The better sensitivity and resolution of CGE-LIF compared to agarose gel electrophoresis has been demonstrated [98]. Capillary electrophoresis is a useful tool for optimization of multiplex PCR reactions by the possibility to obtain quantitative data in form of peak areas/heights [99]. Compared to real-time PCR quantitative results can be achieved in a cheaper way.

2.2 Detection of allergens

In industrialized countries 1-2 % of adults and up to 8 % of children and adolescents are affected by food allergies [100]. The symptoms may range from skin irritations to severe anaphylactic reactions with fatal consequences. Around 90% of the adverse reactions observed have been associated with eight food groups: cow's milk, eggs, fish, crustaceans, peanuts, soybeans, tree nuts and wheat [9, 101]. Besides these major food allergens a broad spectrum of fruits, vegetables, seeds, spices, meats, and latex has been reported to possess allergenic potential [9, 101-102]. The increasing occurrence of food allergies and the possible health risk of unaware consumption of allergenic ingredients yielded in improved labeling regulations in the European Union. Appropriate methods to detect allergens are needed to assure the compliance with these labeling requirements.

2.2.1 Legislation of food allergens in the European Union

Taking into account the recommendations of the Codex Alimentarius Commission [103], the European Commission amended the European Food Labelling Directive 2000/13/EC by a list of ingredients to be labeled [104]. Annex IIIa of Directive 2003/89/EC comprises gluten-containing cereals, crustaceans, eggs, fish, peanuts, soybeans, milk and dairy products (including lactose), nuts, celery, mustard, sesame seeds, sulfite, lupines, molluscs and products thereof. To protect the health of consumers, the declaration of these ingredients has been made mandatory regardless of their amounts in the final product [7, 8].

Provisionally excluded from the Annex IIIa are solely food ingredients mentioned in the Directives 2005/26/EC and 2005/63/EC that are not likely to cause adverse reactions. Examples are highly processed foods like wheat based glucose syrup, nuts used in distillates for spirits or fully refined soy bean oil [105, 106].

Currently, the labeling of allergenic food components and the exceptions to mandatory labeling are specified in Annex IIIa of Directive 2007/68/EC [107].

Accidental, adventitious or technically unavoidable contaminations of allergenic constituents in the final goods are yet ignored by the legislation. Food producers are responsible for such components that are health risks for allergic persons within the due diligence and product liability. Hence, voluntary indication on the label like "may contain…" or "contains traces of…" are widely used by food manufacturers.

2.2.2 Approaches for detection of allergens

Major challenges are the needs to check for the presence of food allergens at extremely low levels and to detect trace amounts of hidden allergens in composite and processed foods [9]. Detection methods for analysis of allergens in food have to provide high specificity and sensitivity, must be reliable, applicable to a wide variety of food matrices and suitable for routine testing. Examples for such approaches are outlined in the following paragraphs.

2.2.2.1 Protein-based methods – ELISA

Allergens are proteins whose routine food analysis is based on immunological detection by specific IgG from mammals using either polyclonal antibodies raised in rabbit or monoclonal antibodies of mouse. Protein-based detection of allergens is mainly done by enzyme-linked immunosorbent assay (ELISA), a technique which is characterized by simple handling, high throughput and the availability of automated systems. Two test principles are used to detect allergenic proteins and peptides in food: the two antibody "sandwich"-ELISA and the competitive ELISA (Figure 4).

With the most frequently applied bilateral sandwich ELISA at least two epitopes are required for detection, thus high specificity is given. Sample proteins are bound to immobilized specific antibodies (capture antibodies), which are typically fixed on microwell/microtiter plates (Figure 4A). After a washing step, further specific enzyme-linked antibodies (detection antibodies) are added detecting the captured antigens. The detection is carried out by addition of chromogenic substrate solution which is enzymatically altered; the resulting change in color is measured spectrophotometrically (Figure 4B). The color intensity of the generated dye is roughly directly proportional to the amount of protein contained in the sample.

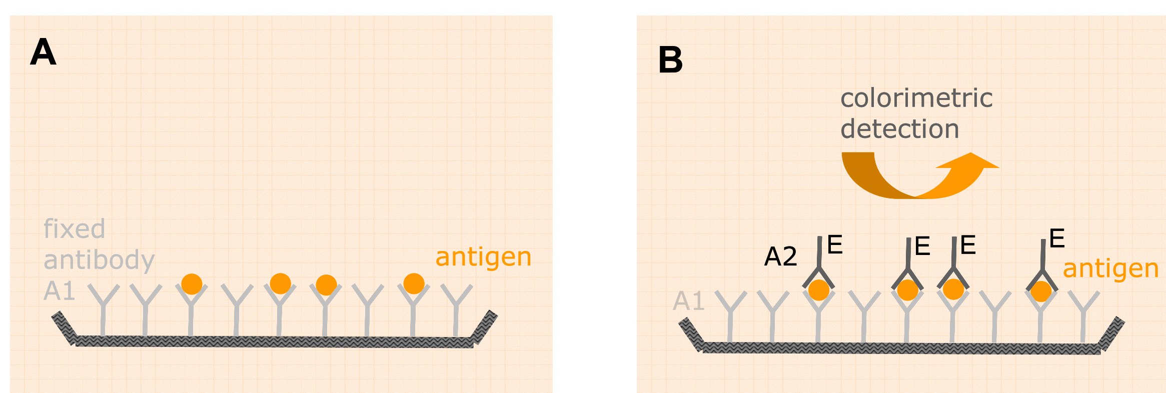

Figure 4 Principle of Sandwich ELISA. Sample antigens are captured by two specific antibodies and colorimetrically detected.

Smaller proteins with only one epitope can be detected with competitive ELISA approaches, for example hydrolyzed milk protein in hypoallergenic baby food. Here, antigens of the sample and immobilized antigens coated on the wells compete for the added specific antibodies (Figure 5A). The more antigens are in the sample, the fewer antibodies can bind to the fixed antigens, and the lower is the enzymatic color reaction (Figure 5B). In this format, the resulting color intensity is inversely proportional to the concentration of the analyte.

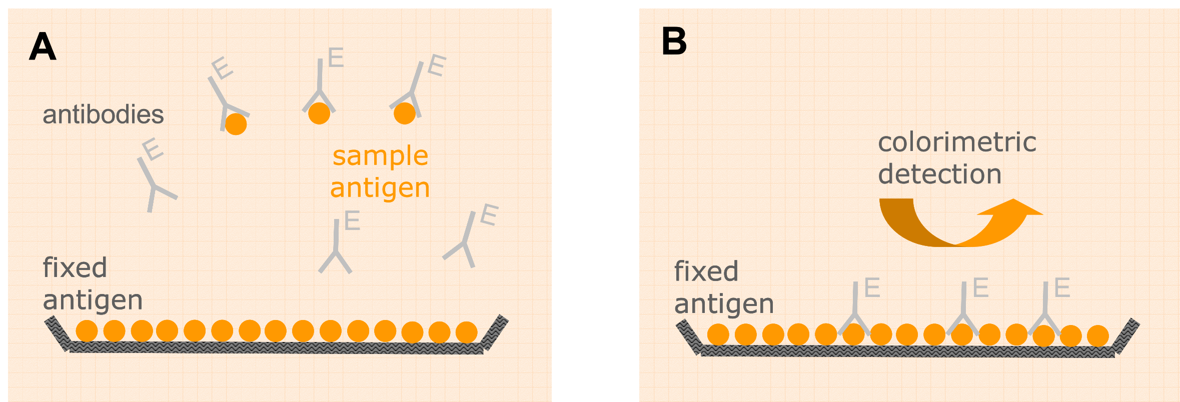

Figure 5 Principle of Competitive ELISA: Competition of fixed and sample antigens to limited enzyme-linked detector antibodies.

2.2.2.2 DNA-based methods

If the allergen itself cannot be targeted, PCR-based methods amplifying specific DNA sequences offer alternative tools [10, 11]. DNA presents a more stable analyte than proteins, is less affected by denaturation and unambiguous sequences allow specific discrimination of closely related species. Sequences specific for allergenic food proteins are targeted by an increasing number of PCR assays. Appropriate PCR assays for the detection and identification of individual food allergens have been developed for cashew [109], celery [110-112], cereals (wheat, barley, rye) [113-115], peanuts [116, 117], pistachio [118] and tree nuts (walnut, hazelnut, pecan nut) [119-124]. Conventional and real-time PCR methods for the detection of soybean, sesame, mustard, peanut, hazelnut and almond have recently been compared [125]. At present, only a few Duplex-PCR systems are known allowing the simultaneous detection of peanut and hazelnut or wheat and barley at the same time [126, 127].

3 Materials and methods

3.1 Materials

3.1.1 Reference materials

Certified maize and soybean powders with defined proportions of GMO-derived material produced by the Institute of Reference Materials and Measurements (IRMM, Geel, Belgium) were purchased from Fluka Chemie AG (Buchs, Switzerland). The flour mixtures contained respectively 0.1, 0.5, 1.0, 2.0 and 5.0 % of transgenic material from the maize lines MON 810, Bt 176, Bt 11 and from Roundup Ready soya. Each set of reference standards included negative control samples free of GMO-derived material.

3.1.2 Rapeseed hybrid molecules and conventional rapeseed material

Rapeseed hybrid amplicons containing one copy of a GMO-specific and a taxon-specific target sequence were synthesized by Moreano et al. [62]. Conventional rapeseed DNA was obtained from Bavarian Health and Food Safety Authority (Landesamt für Gesundheit und Lebensmittelsicherheit, Oberschleißheim, Germany).

3.1.3 Material and food samples for allergen analysis

Nut materials, sesame seeds, ingredients of self-prepared walnut cookies and commercial food samples were purchased from local grocery stores. DNA plant and animal materials used for testing the specificity of the method and spiked samples of chocolate, cookies and pesto used to determine the sensitivity were obtained from the Bavarian Health and Food Safety Authority (Oberschleißheim, Germany). Chocolate samples had been spiked with peanuts (100, 10, 5, 1 and 0.5 mg/kg) and hazelnuts (20, 10 and 5 mg/kg). Cookies spiked with peanuts contained 100, 10, 5, 1 and 0.5 mg/kg of peanuts and pesto spiked with cashew nuts contained 100, 20, 10, 5, 2 and 1 mg/kg of cashew nuts. In all cases samples of the unspiked material were included in the analysis.

3.1.4 Preparation of walnut cookies spiked with different nuts

Nuts ground with a Thermomixer (Vorwerk, Wuppertal, Germany) as well as the other cookie ingredients were analyzed by LPA to ensure the purity of the starting materials. Two doughs containing 25 % wheat flour, 25 % sugar, 25 % butter and 25 % ground nuts (either walnuts only or walnuts spiked with 10 % of peanut, hazelnut, pecan and macadamia) were prepared using a food processor (Braun, Germany). The reference

cookies containing only walnuts and the cookies with all five nuts were baked separately at 180°C for 10 min and ground afterwards. The concentrations of peanut, hazelnut, pecan and macadamia were adjusted in the spiked cookies to 10000, 1000, 100, 10 and 1 mg/kg, respectively, by mixing the corresponding amounts of ground spiked cookies and ground walnut reference cookies in a food processor. The mixtures became fluid due to the high fat contents; however the procedure resulted in visually homogeneous dispersions.

3.1.5 Spiking of pesto samples with cashew nut

Cashew nut material was ground with a mortar and pestle. Commercially available pesto Genovese was homogenized separately for 10 min using a food processor (Braun, Germany) and checked for the absence of cashew DNA prior to spiking. 400 g pesto was spiked with 400 mg of the ground cashew nuts (1000 mg/kg) by gentle manual mixing followed by mechanical mixing for 10 min using the food processor. Successive dilutions of the spiked cashew pesto containing 100, 20, 10, 5, 2 and 1 mg/kg cashew nut were produced with the cashew-free pesto in an analogous manner by manual mixing and homogenization in the food processor.

3.1.6 Oligonucleotides

3.1.6.1 LPA Screening GMO

All probes used for the detection of reference genes, screening and construct-specific elements and event-specific regions were synthesized by TIB MOLBIOL (Berlin, Germany). Primers are included in the MLPA reagents kit (MRC-Holland, Amsterdam, The Netherlands). Sequences of LPA probes and primers are listed in Table 2. In detail, the reference genes in the genome of maize (high mobility group protein (*HMGa*-) gene), soya (lectin (*Le1*-) gene) and rapeseed (acetyl-CoA carboxylase (*BnACCg8*-) gene) were targeted. The CaMV 35S-promotor and the 35S-pat junction (self sequenced by Moreano et al. [62]) were chosen as screening and construct-specific elements. Event-specific regions of the transgenic maize line MON 810 and Roundup Ready soya were also included.

Table 2 LPA probes and primer for GMO detection

Target/ GenBank accession no	**Left probe/ right probe**	**Ligation product [nt]**
Soya Le1-gene/ K00821	5' – **GGGTTCCCTAAGGGTTGGA**CCTTGTTAG TCAAACCACACATAAGAGAGGA – 3' P – 5' –TGGATTTAAACCAGTCAGCACCGTAAGT ATATAGTGA**TCTAGATTGGATCTTGCTGGCAC** – 3'	110
Maize HMGa-gene/ AJ131373	5' – **GGGTTCCCTAAGGGTTGGA**CTCCATCTTC TGTACTAAAGTAGTAGTTGATTGGACTAGAA – 3' P – 5' –ATCTCGTGCTGATTAATTGTTTTACGCGT Gt**TCTAGATTGGATCTTGCTGGCAC** – 3'	114
Rape BnACCg8 gene/ X77576	5' – **GGGTTCCCTAAGGGTTGGA**tgtgtgtgtGAG AATGAGGAGGACCAAGCTCAAGAAAG – 3' P – 5' –AGTGGAGAAAATTCTCAAAGAGGAAGA AGTTAGTTCGA**TCTAGATTGGATCTTGCTGGCAC** – 3'	118
CaMV 35S promotor/ V00141	5' – **GGGTTCCCTAAGGGTTGGA**gtgtgtgtgtgtgt gt*ATCCCACTATCCTTCGCAAGACCCT* – 3' P - 5' –*TCCTCTATATAAGGAAGTTCATTTCATTTGGA* *GAGGACA*gtgtgtgt**TCTAGATTGGATCTTGCTGGCAC** – 3'	130
Junction 35S-pat-gene [62]	5' – **GGGTTCCCTAAGGGTTGGA**tgtg*GGGGATC* *CTCTAGAGTCGACATGTCT* – 3' P – 5' –*CCGGAGAGGAGACCAGTTGAGATTAG*gtgtgtgtgtgt gtgtgtgtgtgtgtgtgtgtgtg**TCTAGATTGGATCTTGCTGGCAC** – 3'	135
Maize MON 810/ AF434709	5' – **GGGTTCCCTAAGGGTTGGA**tgtgtgtgtgtgtgT TTAACATCCTTTG*CCATTGCCCAGCTA* – 3' P – 5' –*TCTGTCACTTTATTGTGAAGATAGTGGAAAA* *GGAAGGT*gtgt**TCTAGATTGGATCTTGCTGGCAC** – 3'	126
Roundup Ready Soya/ AJ308514	5' – **GGGTTCCCTAAGGGTTGGA**tgtgtgtgtgtgt*AATGATGGCA* *TTTGTAGGAGCCACCTT* – 3' P – 5' –*CCTTTTCCA*TTTGGGTTCCCTATGTTTATTTT AACCTGTA**TCTAGATTGGATCTTGCTGGCAC** – 3'	122
Primer R – unlabeled	5´- GTGCCAGCAAGATCCAATCTAGA - 3´	
Primer F – labelled [96]	FAM – 5´-GGGTTCCCTAAGGGTTGGA - 3´	

Uppercase: plantDNA
Uppercase italics: insert DNA
Uppercase bold: primer binding sites
Lowercase: spacer DNA

3.1.6.2 LPA Quantification of maize

Probes used for the detection of the maize high mobility group protein (*HMGa*-) gene as reference gene as well as event-specific regions of the transgenic maize lines Bt 11 (3'-

integration junction), Bt 176 (bar-plant junction) and MON 810 (CaMV junction) were designed using the Beacon Designer 4.0 software (Premier Biosoft Int., USA) and FastPCR software (University of Helsinki, Finland). The synthesis of the probes was done by Biolegio B.V. (Nijmegen, The Netherlands). Primers are included in the MLPA reagents kit (MRC-Holland, Amsterdam, The Netherlands); sequences are listed in Table 2. Sequences of LPA probes as well as GenBank accession numbers of the selected targets are listed in Table 3.

Table 3 LPA probes for GM-maize quantification

Target/ GenBank accession no	Left probe/ right probe	Ligation product [nt]
Maize HMGa-Gen/ AJ131373	5' – **GGGTTCCCTAAGGGTTGGA**tgtgtgtgtgtCCA AGGAGGAAGAGGAGGAAGATGAAGAG – 3'	109
	P – 5' – GAGTCTGACAAGTCCAAGTCGGAGGgt**TCTA** **GATTGGATCTTGCTGGCAC** – 3'	
Maize Bt 11/ AY123624	5' – **GGGTTCCCTAAGGGTTGGA**gtgt*TACATTCA* *AATATGTATCCGCTCATGGAG* – 3'	114
	P - 5' – *GG*ATTCTTGGATTTTTGGTGGAGACCtgtgtgtgt gtgt**TCTAGATTGGATCTTGCTGGCAC** – 3'	
Maize Bt 176/ AJ878607	5' – **GGGTTCCCTAAGGGTTGGA**gtgtgtgtgt*TCCTG* *CCCGTCACCGAGATCTGA*T – 3'	119
	P – 5' – GTTCTCTCCTCCATTGATGCACGCCATgtgtgt gtgtgtgtgt**TCTAGATTGGATCTTGCTGGCAC** – 3'	
Maize MON 810/ AF434709	5' – **GGGTTCCCTAAGGGTTGGA**gtgtgtTCGAAG GACGAAGGACTCTAACGTTTAACA – 3'	104
	P – 5' – TCCTTTG*CCATTGCCCAGCTATCTGT***TCTAG** **ATTGGATCTTGCTGGCAC** – 3'	

Uppercase: plantDNA
Uppercase italics: insert DNA
Uppercase bold: primer binding sites
Lowercase: spacer DNA

3.1.6.3 LPA for the detection of allergens

Probes used for the detection of peanut, cashew, pecan nut, pistachio, hazelnut, sesame, macadamia nut, almond, walnut and brazil nut were designed using the Beacon Designer 4.0 software (Premier Biosoft Int., USA) and FastPCR software (University of Helsinki, Finland). The synthesis of the probes was done by Biolegio B.V. (Nijmegen, The Netherlands). Primers are included in the MLPA reagents kit (MRC-Holland, Amsterdam, The Netherlands); sequences are listed in Table 2. Sequences of LPA probes as well as GenBank accession numbers of the selected targets are listed in Table 4.

Table 4 LPA probes used for the detection of allergens

Target/ GenBank accession no	Left probe/ right probe	Ligation product [nt]
Peanut/ L77197	5'- **GGGTTCCCTAAGGGTTGGA**GCGAGGCA GCAGTGGGAACTC - 3' P - 5' – CAAGGAGACAGAAGATGCCAGAG CC**TCTAGATTGGATCTTGCTGGCAC** - 3'	88
Cashew/ AY081853	5' – **GGGTTCCCTAAGGGTTGGA**CTTATTA GATTAATTCACTGGACTGC - 3' P - 5' – CATGAAGTGAAGCAGTAGTAGAAG**TCT AGATTGGATCTTGCTGGCAC** - 3'	92
Pecan nut/ DQ156215	5' – **GGGTTCCCTAAGGGTTGGA**CACAATC CCTACTACTTTCACTCCCAGGGA - 3' P - 5' – CTCAGGTCGAGACATGAGTCCG GG**TCTAGATTGGATCTTGCTGGCAC** - 3'	96
Pistachio nut/ Y07600	5' – **GGGTTCCCTAAGGGTTGGA**CCTGAA CACGGCGAGCACAAAG - 3' P - 5' – AGGGACTGGTGGAGAAGATCAAAGAC AAgtgtgtgt**TCTAGATTGGATCTTGCTGGCAC** - 3'	100
Hazelnut/ AF136945	5' – **GGGTTCCCTAAGGGTTGGA**GATCACC AGCAAGTACCACACCAAGG - 3' P - 5' – GCAACGCTTCAATCAATGAGGAGGA GAtgtgtgtgt**TCTAGATTGGATCTTGCTGGCAC** - 3'	104
Sesame seeds/ AF240006	5' – **GGGTTCCCTAAGGGTTGGA**gtgtgtTGA AGGGAGAGAAAGAGAGGAGGAGCAA - 3' P - 5' – GAAGAACAGGGACGAGGGCGGATtg tgtgtgt**TCTAGATTGGATCTTGCTGGCAC** - 3'	108
Macadamia nut/ AF161883	5' – **GGGTTCCCTAAGGGTTGGA**CTTA ATCAACCGAGACAACAACGAGAGG - 3' P - 5' – CTCCACATAGCCAAGTTCTTACAGACCA Ttgtgtgtgtgtgt**TCTAGATTGGATCTTGCTGGCAC** - 3'	112
Almond/ X65718	5' – **GGGTTCCCTAAGGGTTGGA**gtgtgtgtgt CCATTACAAGTCTCCACCACCACCAC - 3' P - 5' – CTTCTCCTACTCCTCCAGTCTACTCACC ACCgtgtgtg**TCTAGATTGGATCTTGCTGGCAC** - 3'	116
Walnut/ AF066055	5' – GGGTTCCCTAAGGGTTGGAgtgtgtgtgtGG CACAATCCCTACTACTTTCACTCCCAGAG - 3' P - 5' – CATTAGGTCGAGACATGAGTCCGAGGA AGGtgtgtgt**TCTAGATTGGATCTTGCTGGCAC** - 3'	120
Brazil nut/ M17146	5' – **GGGTTCCCTAAGGGTTGGA**gtgtgtgtgtgt gtgGAGGAGGAGAACCAGGAGGAGTGTC - 3' P - 5' – GCGAGCAGATGCAGAGACAGCAGgtgtgt gtgtgtgtgtg**TCTAGATTGGATCTTGCTGGCAC** - 3'	124

Capitals: plant DNA
Bold capitals: primer binding site
Lowercase fonts: spacer DNA

3.1.6.4 Real-time PCR for the detection of cashew-DNA

Primers for conventional PCR as well as primers and probe for real-time PCR detection of a DNA sequence encoding part of an important cashew nut (*Anacardium occidentale L.*) allergen (Ana o3) of the 2S albumin family [128] were designed using the Beacon Designer 4.0 software (Premier Biosoft Int., USA). Amplicon lengths are 529 bp for the conventional PCR system and 103 bp for the real-time PCR system. Synthesis of primers and probe was done by TIB MOLBIOL (Berlin, Germany); sequences are listed in Table 5.

Table 5 Primers and probes for the specific detection of cashew

Primer/ Probe	Sequence	Amplicon length [bp]
Cashew F	5' – TCCTCCTCCTGGTGGCTAAC – 3'	529
Cashew R	5' – ATTACATAGCCTCAATCACTGACAC – 3'	
Cashew RT F	5' – CCATGAAGTGAAGCAGTAGTAGAAG – 3'	103
Cashew RT R	5' – GACTCTGTGCTGATTCTACTACTC – 3'	
Cashew RT probe	FAM – TTCACAACTCTGGCGTTTAAGCTGGA – TAMRA	

3.2 Methods

3.2.1 DNA extraction

3.2.1.1 Wizard method

The Wizard® extraction was performed by adding 860 µL extraction buffer [10 mM Tris-HCL, 150 mM NaCl, 2 mM EDTA, 1% (w/w) sodium dodecyl sulfate (SDS), pH 8.0], 100 µL guanidine chloride (5 M) and 40 µL proteinase K (20 mg/mL) to each sample (300 mg). After incubation at 60°C for 3 hours, the mixture was centrifuged at 12.000 g for 10 min. The supernatant (500 µL) was transferred to another tube, mixed with 5 µL RNase A (10 mg/mL) and incubated at 60°C for 5 min. The extracted DNA was purified according to the Wizard® isolation protocol [129], using the respective DNA binding resin and mini-columns (Promega, Madison, USA).

3.2.1.2 CTAB method

DNA extraction was performed by a CTAB protocol adapted to the fatty nuts matrix. Each sample (2 g) was mixed with 10 mL CTAB-extraction buffer [2 % (w/v) cetyltrimethylammoniumbromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-OH/HCl] and

30 µL proteinase K (20mg/mL) in an 50 mL tube and incubated at 65°C overnight. After 5 min centrifugation at 5000 g, 1000 µL supernatant was transferred to an 1.5 mL tube and centrifuged again at 14000 g for 5 min. 500 µL chloroform/isoamylalcohol (Ready Red™) was mixed with 700 µL supernatant and centrifuged at 16000 g for 15 min. 500 µL supernatant was added to 500 µL isopropanol (stored at -20°C) and incubated at RT for 30 min. After 15 min centrifugation at 16000 g the supernatant was removed, the pellet was washed with 500 µL ethanol (70 %; stored at -20°C) and centrifuged 5 min at 16000 g. After removal of ethanol the pellet was dried 1 h at 50°C and afterwards diluted in 100 µL TE-buffer (1x). Additionally, the DNA extracts were purified using spin filter columns.

3.2.2 Photometric DNA analysis

DNA concentrations for GMO analysis were determined spectrophotometrically at 260 nm using a UV/VIS spectrometer (Kontron, Neufahrn, Germany). DNA purity was determined by measuring additionally at 280 nm and calculating the ratios (260/280).

$$c_{DNA}\,[ng/\mu L] = F \cdot C_{DNA} \cdot \Delta ABS$$

F = dilution factor

C_{DNA} = 50 (constant for double stranded DNA)

ΔABS = absorption against H_2O ($0.1 \leq \Delta ABS \leq 1.0$)

3.2.3 Fluorometric DNA analysis

DNA concentrations of material used for allergen detection methods were determined fluorimetrically at 520 nm using PicoGreen® dsDNA quantification reagent (Invitrogen, Karlsruhe, Germany) on a Tecan GENios™ plus reader (Männedorf, Switzerland); excitation wavelength 480 nm. Samples were diluted 1:10 with Picogreen working solution (1:400 dilution of stock dye in TE). DNA standards from λ-DNA were run concurrently and double-stranded DNA concentrations were quantified using the Tecan Magellan™ software package.

3.2.4 Electrophoresis

DNA extracts or PCR products were analyzed using 2 % agarose gels (UltraPure Agarose, Invitrogen, Karlsruhe, Germany). DNA extracts or PCR products were mixed with loading buffer [TBE buffer (pH 8.0, 45 mM Tris/ boric acid, 1mM Na-EDTA), 40 % glycerine, 2.5 g/L bromphenol blue sodium salt] in a proportion 1:5 and loaded into the ethidium bromide stained gels. Electrophoresis was performed in TBE buffer at 120 mV and room

temperature until an appropriate separation of the DNA was achieved. Gels were digitized using a CCD camera-based documentation system GelDoc 1000 and the respective Multi Analyst 1.0.2 software (Bio Rad, München, Germany).

3.2.5 Ligation-dependent probe amplification

The LPA reaction was essentially carried out as described by Schouten et al. [96]. Hybridization was performed overnight in 0.5 mL reaction vessels using a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) and 100 ng DNA sample. After 5 min of DNA denaturation at 98°C 1.5 µL of MLPA buffer and 1.5 µL of a mixture of the synthetic probes, containing each LPA probe of the used system (see Table 6), were added and held at 60°C for 16 h. Ligation reaction was performed at 54°C for 15 min adding 3 µL Ligase-65 buffer A, 3 µL Ligase-65 buffer B, 25 µL H_2O and 1 µL Ligase-65 (MRC-Holland, Amsterdam, The Netherlands). After ligation, reaction mixes were heated for 5 min at 98°C to inactivate the enzyme.

For amplification of ligation products 10 µL Polymerase mix with primers, dNTPs, buffer and Polymerase enzyme of the MLPA kit (MRC-Holland, Amsterdam, The Netherlands) were added to 40 µL ligation reaction at 60°C. 35 amplification cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec were followed by a final step of 20 min at 72°C and cooled down to 4°C.

Table 6 Concentrations of synthetic probes in the LPA mixture

LPA system	Probe	Concentration [fmol]
Screening GMO	*HMGa*- gene, *Le1*- gene,	0.2
	BnACCg8- gene, competitor maize (*HMGa*)	1
	CaMV 35S promoter, 35S-pat junction, RRS, competitor soya (*Le1*)	2
	MON 810	3
Maize quantitation	*HMGa*- gene,	0.2
	MON 810	2
	Bt 11, Bt 176	3
	Competitor maize (*HMGa*)	5
Allergens	peanut, cashew nut, pecan nut, pistachio, hazelnut, sesame, macadamia nut, almond, walnut and brazil nut	1

3.2.6 Fragment length analysis

Fragment length analysis was performed on an ABI PRISM® 310 Genetic Analyzer using capillaries (47 cm) and polymer (POP-6™ Performance Optimized Polymer); reagents were obtained from Applied Biosystems (Forster City, USA). One microliter of the PCR product (pure or diluted) was mixed with 0.3 µL size standard (GeneScan®-500 [TAMRA]™) and 14.7 µL Hi-Di™ formamide. Prior to analysis, DNA was denatured at 94°C for 3 min and cooled down on ice. Electrokinetic injections were performed at 15 kV for 5 sec. Electrophoretic separations were run at 60°C and 15 kV.

3.2.7 Sequencing

Ligation products were generated and amplified separately prior to sequencing. Primers used for sequencing were identical to those listed in Table 1; FAM-labeling did not interfere with cycle sequencing. Amplified products were cleaned up using a PCR purification kit (QIAquick, Qiagen GmbH, Hilden, Germany) and used as template for the sequencing PCR. This reaction was performed using a BigDye, Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). One reaction mix (20 µL) contained 2 µL 5x buffer, 4 µL RR-mix, 2 µL primer (10 pmol), 8 µL H_2O and 4 µL template. Reaction conditions were as follows: initial denaturation (1 min at 96°C), 30 cycle denaturation steps (10 sec at 96°C) and primer annealing (5 sec at 56°C), and a final step (4 min at 60°C).

Purification of the PCR products was carried out following amplification. PCR products (10 µL) were mixed with 16 µL H_2O, 4 µL Na-acetate (3M) and 50 µL EtOH (100%) in a 1.5 mL reaction vessel and centrifuged at 15.000 rpm for 15 min. EtOH was removed carefully without damaging the precipitated DNA pellet. The pellet was vortexed with 50 µL EtOH and centrifuged at 15.000 rpm for 5 min. After carefully removing EtOH, the pellet was allowed to dry at 50°C for 1 hr. Finally, DNA was dissolved in 20 µL H_2O.

Sequencing of the diluted PCR products (10 µL H_2O + 6 µL purified DNA) was carried out on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Forster City, USA) using 47 cm capillaries and POP-6™ Performance Optimized Polymer. Electrokinetic injections were performed at 2 kV for 30 sec. Runs were carried out at 50°C and 15 kV.

3.2.8 Conventional PCR

Conventional PCR was performed on a MWG thermocycler (MWG-Biotech, Ebersberg, Germany) to generate a 529 bp amplicon of the target region. Reaction mixes (25 µl) containing 1x AmpliTaq® Gold reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each

deoxynucleotide triphosphate and 2 units AmpliTaq Gold® DNA polymerase (PE Applied Biosystems, Weiterstadt, Germany) and 0.4 µM of each primer Cashew F and R, respectively. Reactions were run over 40 cycles according to the following temperature program: initial denaturation (8 min at 95°C), cycle denaturation (30 s at 95°C), primer annealing (30 s at 58°C), cycle elongation (30 s at 72°C) and final elongation (5 min at 72°C). Cycle sequencing was performed in order to confirm the sequence identity of the cashew reference DNA.

3.2.9 Real-time PCR

The real-time PCR for the specific detection of cashew nut was performed using an ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Forster City, USA). Reactions were run in 25 µl total volume containing the TaqMan™ Universal PCR Mastermix (Applied Biosystems, Forster City, USA), final concentrations of 0.3 µM of each primer and 0.2 µM of the probe and 5 µl of the pure or 1:10 diluted sample DNA extract. Fluorimetric measured DNA concentrations of the samples used for real-time PCR analysis ranging from 2 ng/µl to 20 ng/µl. Reactions were run over 45 cycles according to the following temperature program: Uracil-N-Glycosylase (UNG) decontamination (2 min at 50°C), initial denaturation (10 min at 95°C), cycle denaturation (15 s at 95°C), primer annealing, elongation and data collection (60 s at 60°C). Collected data (C_T values) were exported to a Microsoft Excel file for evaluation. Additional positive and negative control reactions, as well as extraction and inhibition control samples were performed.

Table 7 Reaction components for the detection of cashew by real-time PCR

Component	Initial concentration	Final concentration	µL/ PCR	µl/ PCR inhibition
TaqMan™ Universal PCR Mastermix	2x	1x	12.5	12.5
Cashew RT F	10 µM	0.3 µM	0.75	0.75
Cashew RT R	10 µM	0.3 µM	0.75	0.75
Cashew RT probe	10 µM	0.2 µM	0.5	0.5
Water			5.5	4.5
PCR product V_8	100 copies/ µL	4 copies/ µL		1
Template			5	5

In order to compare LPA results with established methods for the detection of allergens commercially available test kits were purchased. SureFood® Allergen Kits for the qualitative detection of DNA from hazelnut (Art. No. S3102) and peanut (Art. No. S3103)

from Congen Biotechnology GmbH (Berlin, Germany) were used for real-time PCR analysis following the respective kit protocols. The limits of detection for both kits were indicated to be 10 copies of genomic DNA.

3.2.10 Enzyme linked immunosorbent assay (ELISA)

In order to compare LPA results with established methods for the detection of allergens commercially available test kits were purchased. Ridascreen® enzyme immunoassays for the quantitative analysis of hazelnut (Art. No. R6801) and peanut (Art. No. R6201) were acquired from R-Biopharm AG (Darmstadt, Germany) and carried out as described in the respective kit manual. The limit of detection as indicated by the manufacturer were 2.5 mg/kg hazelnut and peanut, respectively.

4 Results and discussion

4.1 LPA Screening GMO

In the European Union regulations have been implemented containing harmonized provisions for the risk assessment and authorization of genetically modified organisms (GMO) as well as for traceability, labeling and post-marketing surveillance of the use of GMO in the food and feed chain [1, 2]. To control the compliance with these legal provisions appropriate methods for detection and quantification of GMO are required. The European Commission's Reference Laboratory provides official methods for the event-specific detection and quantification of material from authorized GMO. Major analytical challenges arise from the continuous need to develop and to establish further assays (i) for forthcoming authorized GMO, (ii) for GMO lines close to authorization, which have benefited from a favorable risk evaluation by the European Food Safety Authority, and (iii) for unauthorized GMO. This requires flexible surveillance systems which can be easily expanded.

Multiplex assays allowing the simultaneous detection of several GMO in a single PCR reaction have been developed [31, 36, 82-87]. Some of these approaches use similar primers for the detection of different targets to overcome the difficulties of simultaneous amplification of multiple oligonucleotides in one single reaction [31, 82]. Disadvantages of these approaches using gel electrophoresis for separation are the lack of sensitivity, the long amplicon lengths and the length differences of the targets, which pose a problem when applying the method to the analysis of processed foods [84]. At present the simultaneous amplification using nine primer pairs for the detection of different GM maize events represents the most comprehensive multiplex PCR method [87]. In addition, applications for identification of GMO in food have been introduced using sensitive detection via microarray technology [42, 88-89, 91-95]. However, multiplex PCR systems are limited by interactions between the different primer pairs and their possible non-competitive amplification. Further, the possibilities for subsequent alterations of targets or incorporation of additional primers for the detection of further target sequences in assembled multiplex assays are restricted.

This chapter describes the application of a ligation-dependent probe amplification (LPA) technique for the simultaneous detection of DNA from several GMO in a single reaction. Ligation-dependent PCR was originally introduced to allow the detection of nucleic acid sequences [130-132]. First applications in the field of medical diagnostics allowed the detection and the relative quantification of up to 40 – 50 target sequences in a single assay [96, 133-136]. The technique does not amplify the target sequences itself, but is rather based on the amplification of products resulting from the ligation of bipartite

hybridization probes. Automated separation and detection of amplified products via capillary electrophoresis simplifies and reduces the extent of post-PCR work-up significantly. The suitability of this method for the event-specific detection and relative quantification of two GMO has been demonstrated for two GMO using commercially available maize and soya standards [97].

The strategy provides a flexible system that can be complemented with further hybridization probes to broaden the range of target sequences to be detected. Therefore, the objective of this study was to develop an approach allowing the simultaneous detection of several targets corresponding to different levels of specificity in a one-tube assay. Synthetic oligonucleotides were designed to detect (i) reference genes in the genome from maize, soya and rapeseed, (ii) the CaMV 35S-promotor as screening element, (iii) the construct-specific 35S-pat junction, and (iv) the event-specific regions of the transgenic maize line MON 810 and of Roundup Ready soya. By detection of screening elements in combination with construct-specific targets and suitable reference genes a profiling-like strategy suitable (and expandable) for analysis of a broad spectrum of GMO should be developed.

4.1.1 Design of an LPA system for GMO screening in food

For each of the target sequences to be detected the design of two probes containing the respective target-specific hybridization sites as well as identical primer binding sites (PBS) at their 5'- or 3'- ends is required. The LPA system designed for this study uses synthetic oligonucleotides for hybridization as described by Moreano et al. [97]. Sequences of LPA probes and primers are listed in Table 2.

Length and base composition of the targeted hybridization sites were chosen to achieve similar thermodynamic properties. Theoretical melting temperatures of approximately 66°C of each hybridization site assure a specific ligation reaction performed at 60°C. As described in 2.1.2.3, the choice of the size of the target sequence influences the detectability of DNA. Degradation of DNA in the course of food processing negatively affects the detection efficiency, especially when long sequences are the targets. This results in false negative results when analyzing samples of processed foods and feeds. Therefore, the lengths of hybridization regions were selected to be at most 71 nucleotides (nt) to assure the accurate detection even in processed products showing highly degraded DNA.

The targets were selected to create an LPA screening system allowing detections of GMO at different levels of specificity: The promoter of the Cauliflower Mosaic Virus (CaMV 35S) used in most transgenic crops to activate foreign genes was targeted as screening element (regulatory sequence). Targeting the junction region between the CaMV 35S

promoter and the *pat*-gene allows the construct-specific detection of different rapeseed LibertyLink™ lines (self-sequenced by Moreano et al. [62]). Additionally, the event-specific detection of maize MON 810 [34] and Roundup Ready soya [38] by targeting junction regions between the 35S promoter and the plant DNA in each GM line permits quantitative assessment of GMO [97]. Suitable reference genes in the genome of maize (*HMGa*)-gene, soya (*Le1*)-gene and rapeseed-specific acetyl-CoA carboxylase (*BnACCg8*-) gene were selected.

The primer sequences described by Schouten et al. [96] for MLPA analysis were tested for their suitability to analyze GMO in foods. The specificity was evaluated on the basis of database enquiry via NCBI GenBank and PCR using DNA of maize, soya, rapeseed and rice (data not shown).

The use of spacer sequences between PBS and hybridization sites rendered ligation products with lengths characteristic for each of the target DNA. Differences of 4 nt in length have been shown sufficient for unequivocal determination of the amplification products using POP-6™ polymer. Compared to POP-4™ polymer applied in former studies [97], better resolution and separation of the individual peaks could be achieved by keeping the peak width. Simple repeats of GT bases were used for the spacer sequences to avoid intra- and intermolecular hybridizations. Competitors were used to reduce off-scale signals of the reference targets to the dynamic range of the capillary sequencer. These competitor oligonucleotides only consist of the hybridization part of the right probe without the primer binding site, and thus cannot be amplified during PCR [135].

4.1.2 Evaluation of target specificity

The performance of the LPA system was examined using (i) DNA isolates from non-transgenic rapeseed, maize and soya, (ii) certified reference flour standards from maize MON 810 and Roundup Ready soya with defined proportions between 0.1 and 5 % GMO-derived material and negative controls free of transgenic material, and (iii) synthesized hybrid amplicons containing copies of the rapeseed-specific target and of the LibertyLink™-specific junction region P-35S/*pat* used as rapeseed DNA-standards (10^5 copies/µL). The DNA extracts were adjusted to 50 ng/µL and different mixtures are prepared to simulate composed food products with different GMO proportions.

Target specificity evaluation of the developed LPA-system was shown to be suitable for the simultaneous detection of all target sequences in a single reaction, depending on the composition of the template DNA used in each experiment. As illustrated in Figure 6A, analysis of a mixture containing 5 % maize MON 810 and Roundup Ready soya together with hybrid molecules resulted in seven signals corresponding to the reference genes (*HMGa*, *Le1* and *BnACCg8*), the event-specific target sequences, the CaMV 35S

Promotor and the targeted junction 35S/*pat*-gene. Compared to a sample mixture containing 5 % maize MON 810 and Roundup Ready soya and conventional rapeseed DNA instead of hybrid molecules resulted in the lack of the signal according to the 35S/*pat* junction region, as shown in Figure 6B. No false-positive signals were obtained with all tested mixtures, or using different DNA extracts from other plant and animal species (data not shown).

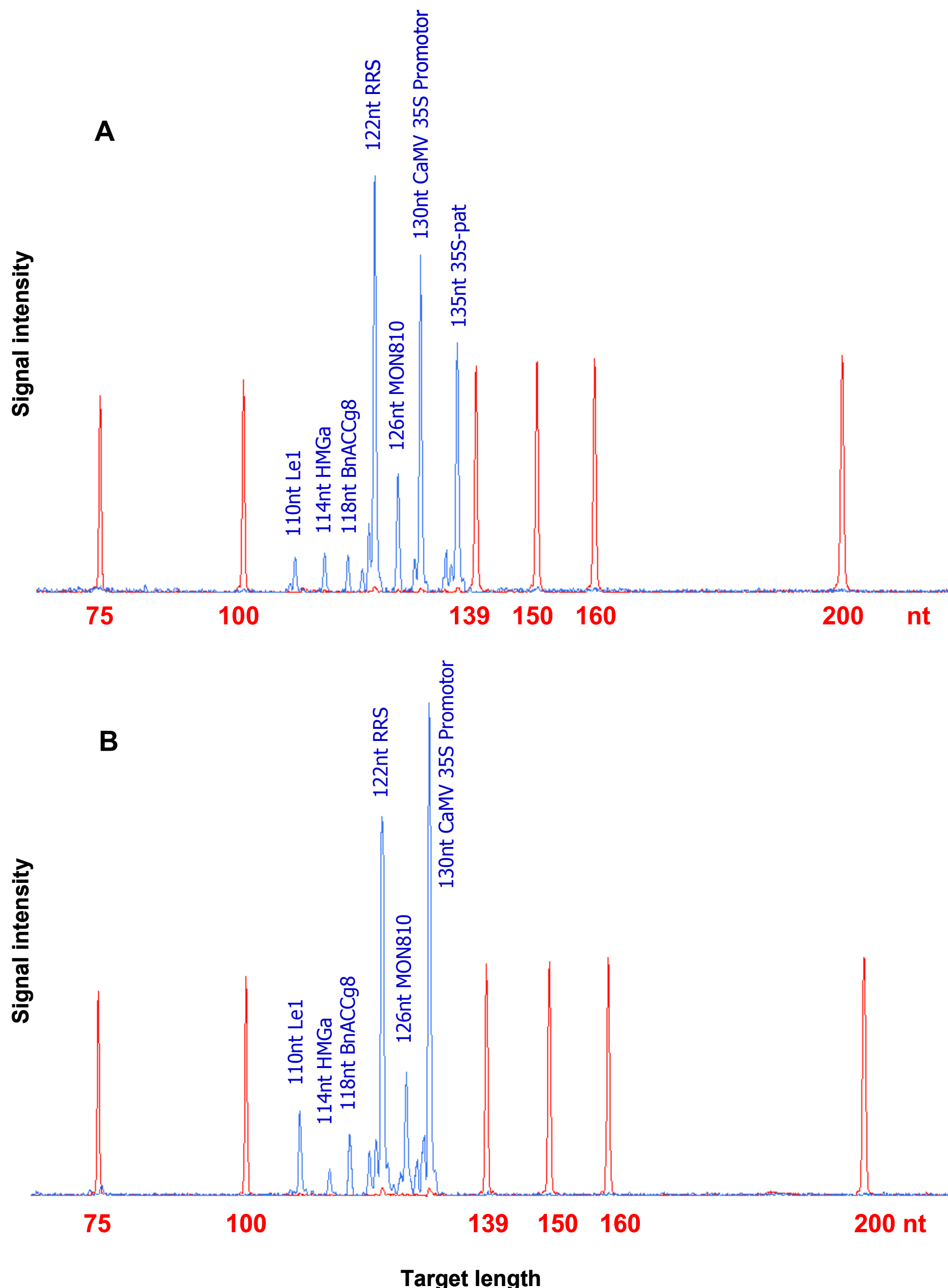

Figure 6 Scope and specificity of the LPA GMO screening system. (A) Simultaneous detection of all seven targets in one reaction in a mixture containing soya (5 % Roundup Ready), maize (5 % MON 810) and hybrid molecules that include both the rapeseed-specific target of BnACCg8-gene and the transgene construct P35S-pat (105copies). (B) Electropherogram of a sample with 5 % Roundup ready soya, 5 % MON810 maize and conventional rapeseed DNA resulted in the lack of the P-35S-pat target. Non-assigned signals correspond to the used size standard.

The fragment lengths for each target were highly reproducible (SD ± 0.2 nt) with slight shifts (≤ 1 nt) of the absolute length value (Table 8). This well-known effect arises from differences in the mobility of length standards and analyzed fragments caused by different labelin dyes and by the structure and base composition of the DNA itself [137, 138].

Table 8 Amplicon length and retention time of LPA Probes for GMO detection

Target	Amplicon length [nt]	Retention time [nt]
Soya *Le1*-gene	110	108.88 ± 0.23
Maize *HMGa*-gene	114	113.92 ± 0.15
Rape *BnACCg8*-gene	118	117.26 ± 0.28
Roundup Ready Soya	122	122.35 ± 0.19
Maize MON 810	126	126.38 ± 0.22
CaMV 35S junction	130	130.12 ± 0.18
Junction 35S-pat	135	136.00 ± 0.23

* mean of 12 to 18 single values from 4 different experiments/ days

If high signal intensities are achieved, slight peaks appear at intervals of 1 and 2 nucleotides away, respectively. Firstly, the set-up of the PCR was chosen to promote the non-templated addition of preferable adenine nucleotide by the DNA polymerase using 3-step PCR conditions plus 20 min final extension at 72°C [137, 139], but the true PCR product cannot be eliminated completely. To confirm the identity of the signals, cycle sequencing of the separately amplified ligation products was performed. Secondly, minor signals can result from slippage of DNA polymerase that produces PCR stutter products [140]. Another reason may be the presence of impurities in the synthesized oligonucleotides[141, 142] used in the ligation reaction. A new synthesis of especially long oligonucleotides led to a significant reduction of the n-1 and n-2 signals, respectively (cf. 4.3.2).

4.1.3 Assessment of the sensitivity of the LPA screening system

The suitability of this system to detect trace amounts of GMO was assessed using certified reference materials with defined GMO proportions. As shown exemplarily for Roundup Ready soya in Figure 7, the optimization of probe and competitor concentrations as well as of the reaction conditions allowed the positive detection of a sample containing 0.1 % GMO (Figure 7A). In addition to the taxon-specific signal, the signals corresponding to the event-specific target and to the screening element are detected.

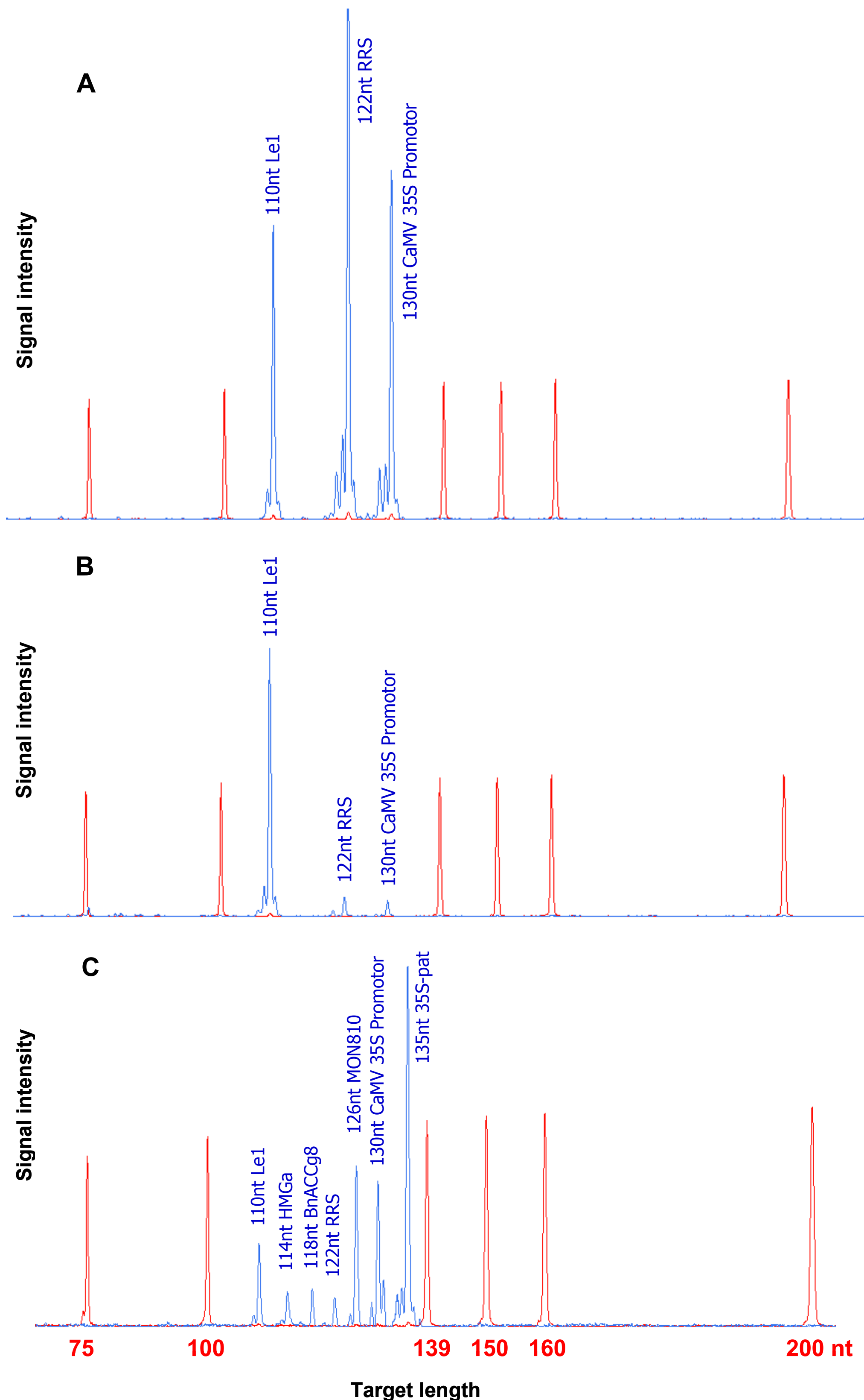

Figure 7 Assessment of the sensitivity of the LPA screening system for GMO. (A) Electropherogram of a sample containing 5 % Roundup Ready soya DNA showing the three corresponding signals, (B) 0.1% Roundup Ready soya DNA sample was tested positive on screening element CaMV 35S Promotor and event-specific target. (C) Detection of 0.1 % Roundup ready soya in presence of 5 % MON 810 maize and hybrid molecules. Non-assigned signals correspond to the used size standard.

The detection of GMO at threshold levels was not affected in samples containing other target sequences even at high levels. The presence of 0.1 % Roundup Ready soya in the presence of 5 % maize MON 810 and rapeseed hybrid amplicons could be confirmed (Figure 7C). Further experiments are necessary to determine the practical limit of detection by analysis of different food matrices.

To determine the robustness of the method, the experiment was slightly varied by performing the protocol with a different operator, simulating influences of small assay modifications/deviations as described by the ENGL method performance requirements for GMO analysis [108]. No differences in the determination of sensitivity could be observed between the two operators.

4.1.4 Summary

The LPA assay represents a useful analytical strategy for screening different genetically modified organism in foods. The simultaneous detection of screening elements in combination with construct-specific targets and suitable reference genes provides a profiling-like strategy for GMO analysis including unauthorized and unknown events. Relative limits of detection were determined to 0.1 % of transgenic material by analysis of certified reference standards. Assessment of the sensitivity demonstrated the definite detection of traces of 0.1 % GMO that remained unaffected even in samples containing all targets in variable weight proportions. The modular system allows the extension to further target sequences of interest, both relevant taxon-specific regions and probes for the detection of other screening elements like the NOS terminator.

The use of synthetic oligonucleotides as probes and the employment of classical thermocycler and detection methods enable the implementation of the technique in commonly equipped laboratories. The work effort of LPA is approximately comparable to real-time PCR analysis whereas time-consuming hybridization reaction must be well arranged. However, multiple conclusions can be obtained by performing a single LPA reaction. In addition, results can be achieved in a cheaper way due to the use of less reagents and consumables by the multiplex approach. Detection using capillary electrophoresis via laser-induced fluorescence represents a very sensitive detection method at labeling threshold levels and enables automated analysis of the samples without further confirmation/verification of the obtained results. A validation of the developed assay still needs to be performed to evaluate the method, e.g. in comparison to the real-time PCR technique.

4.2 Relative quantification of different genetically modified maize lines

The profoundly amended regulations on the use of GMO within the food and feed chain implemented surveillance requirements that will pose a challenge for monitoring and reference laboratories [1, 2]. Official methods to control the compliance with these legal provisions are provided by the European Commission's Reference Laboratory for the event-specific detection and quantification of material derived from authorized GMO lines. Beyond this, appropriate assays have to be developed and established for the screening, identification and quantification of forthcoming authorized GMO lines and unauthorized GMO. The steadily growing number of analytical targets requires flexible and extendable surveillance tools.

Currently, real-time PCR methods as standard techniques for quantitative GMO analysis are run in mono- or duplex formats [65, 78, 145], whereas multiplex applications are restricted by interactions between the diverse primer and probes and the limited number of simultaneously detectable reporter dyes. Multiplex assays allowing the simultaneous detection of several GMO in a single PCR reaction have been developed [31, 36, 82-87], but the lack of sensitivity and unavailable quantitative results are drawbacks of these approaches. Alternative applications for identification of GMO in food have been introduced using sensitive detection via microarray technology [42, 88-89, 91-95]. However, multiplex PCR systems are limited by interactions between the different primer pairs and their possible non-competitive amplification prior to on-chip detection. Normalization of signals from recombinant targets and respective reference targets are required for quantitative GMO assessment. Furthermore, the possibilities for subsequent alterations of targets or incorporation of additional primers for the detection of other target sequences in assembled multiplex assays are restricted.

The following approach describes the development of a flexible LPA system for the simultaneous event-specific quantification of different GMO maize lines. The application of ligation-dependent probe amplification for the quantitative analysis of DNA from two GMO has been exemplarily shown for Roundup Ready soya and for maize MON 810 on the basis of calibration curves [97]. Sensitivity, specificity and linear correlation of the LPA system for GMO analysis should be demonstrated using certified reference materials with different GMO proportions. The objective of this study was to show the suitability of the method for the simultaneous event-specific detection and relative quantification of different recombinant maize lines.

4.2.1 Design of the LPA system

For the detection of each target sequence two probes had to be designed containing the respective target-specific sequence and the primer binding sites at their 3'- and 5'- ends, respectively. Relevant criteria as described in 4.1.1 have been considered for the design of synthetic probes for hybridization.

Similar thermodynamic properties with theoretical melting temperature (T_M) of about 65°C were preferably chosen for the hybridization sites of the recombinant targets and the reference target. Selected sequences should be at the highest level of specificity for quantification purposes. This implied an event-specific assay design to assure unequivocal differentiation of the single GM maize events. The choice of probe position at the integration site was restricted to a given DNA section and therefore had to be in agreement with different DNA properties and base compositions of the individual targets. Especially, the theoretical T_M for the hybridization region of Bt 11 probes was calculated 3 – 4°C lower than for the other targeted DNA regions. Short hybridization sites of 52 - 56bp length guarantee sensitive detection and overcome difficulties arising from DNA degradation in processed foods (cf. 2.1.2.3). Furthermore, nearly constant lengths of the hybridization regions of all targets were chosen to avoid distortion of relative quantification results (cf. 2.1.4.3) [77].

Sequences of LPA probes for simultaneous relative quantification of different recombinant maize lines are listed in Table 3. LPA probes were designed to target the junction region between the 35S promoter and the plant DNA of MON 810 [33], the 3'- integration junction between the *pat*- gene and the plant DNA of maize Bt 11 [27] and, the 3'- integration site of *bar*- gene flanking maize genomic DNA of Bt 176 [29]. The maize reference gene (*HMGa*-) has been shown to be suitable for quantitative analysis [78]. The primer sequences adapted from Schouten et al. [96] were tested in 4.1.1 to be suitable for GMO analysis in foods and are listed in Table 2. Spacer sequences to assure characteristic lengths of the probes were required for the simultaneous separation of PCR products via capillary electrophoresis. Length differences of 5 nt are reached by simple GT repeats to avoid intra- and intermolecular hybridizations.

A maize (*HMGa*) competitor (for sequence details see Table 3) was used to reduce the signal intensity, caused by the manifold targets of the reference gene compared to the traces of recombinant targets, to the dynamic range of the capillary sequencer. These competitor oligonucleotides consist of the hybridization part of the right probe without the sequence of the primer binding site, and thus cannot be amplified during PCR [133]. Especially for the relative quantification of GMO, competitors are an essential tool to avoid detector overload of the instrument and thus permit the quantification of peak

areas/heights. Detection via laser-induced fluorescence presents a very sensitive device, but the dynamic range is comparatively small.

4.2.2 Target specificity and sensitivity estimation

The performance of the LPA system for the simultaneous quantification of different recombinant maize lines was examined using certified reference flour standards from maize MON 810, Bt 11 and Bt 176 with defined proportions between 0.1 % and 5 % GMO-derived material and negative controls free of transgenic material. The DNA extracts were adjusted to 50 ng/µl and different mixtures were prepared to simulate composed food products with different GMO proportions.

Target specificity evaluation of the LPA system confirmed the suitability of this technique for the simultaneous detection of all targets in a single reaction, depending on the compilation of the sample. No false-positive signals were obtained with all tested mixtures, or using different DNA extracts from other plant and animal species (data not shown). As shown in Table 9, the fragment lengths for all targets were highly reproducible (SD ± 0.2 nt) with slight shifts (≤ 1 nt) of the absolute length value. This well-known effect results from varying mobility of the analyzed PCR products and the length standards caused by different labeling dyes and by the structure and base composition of the DNA itself [135, 136]. However, the identity of the analyzed fragments was confirmed by cycle sequencing of the separately amplified ligation products.

Table 9 Amplicon lengths and retention times of LPA Probes used for GM-maize quantitation

Target	**Amplicon length [nt]**	**Retention time [nt] ***
Maize *HMGa*-gene	109	108.85 ± 0.19
Maize MON 810	104	103.12 ± 0.19
Bt 11 maize	114	114.37 ± 0.18
Bt 176 maize	119	119.34 ± 0.15

* means of 25 to 40 single values from three different experiments/days

The sensitivity was evaluated using certified reference materials with defined GMO proportions to verify the correctness of the system to detect even trace amounts of transgenic DNA. As illustrated in Figure 8, the optimization of probe and competitor concentrations permitted the positive determination of samples containing either 0.1 % of (A) MON 810, (B) Bt 11, or (C) Bt 176 DNA. In addition to the maize *HMGa* reference target, signals corresponding to the respective event-specific recombinant targets were detected. This remained unaffected by the presence of DNA from different sources or

other target sequences as already shown in former studies (cf. 4.1.3). The application of 5 fmol maize competitor oligonucleotides and limitation of the maize HMGa probes to 0.2 fmol was essential to receive the appropriate sensitivity for the recombinant targets. However, the signal intensity of the Bt 11 target (Figure 8B) still remained too small for quantitative interpretation of the 0.1 % standard and this will limit the range of quantitative analysis.

Minor signals at 1 - 2 nt distance were observed at the high-resolution electropherograms in particular with higher signal intensities. This may be caused by different effects as described in 4.1.2. A combination of PCR set-up, polymerase features/faults and impurities of the synthetic oligonucleotides could be responsible [137, 139-142].

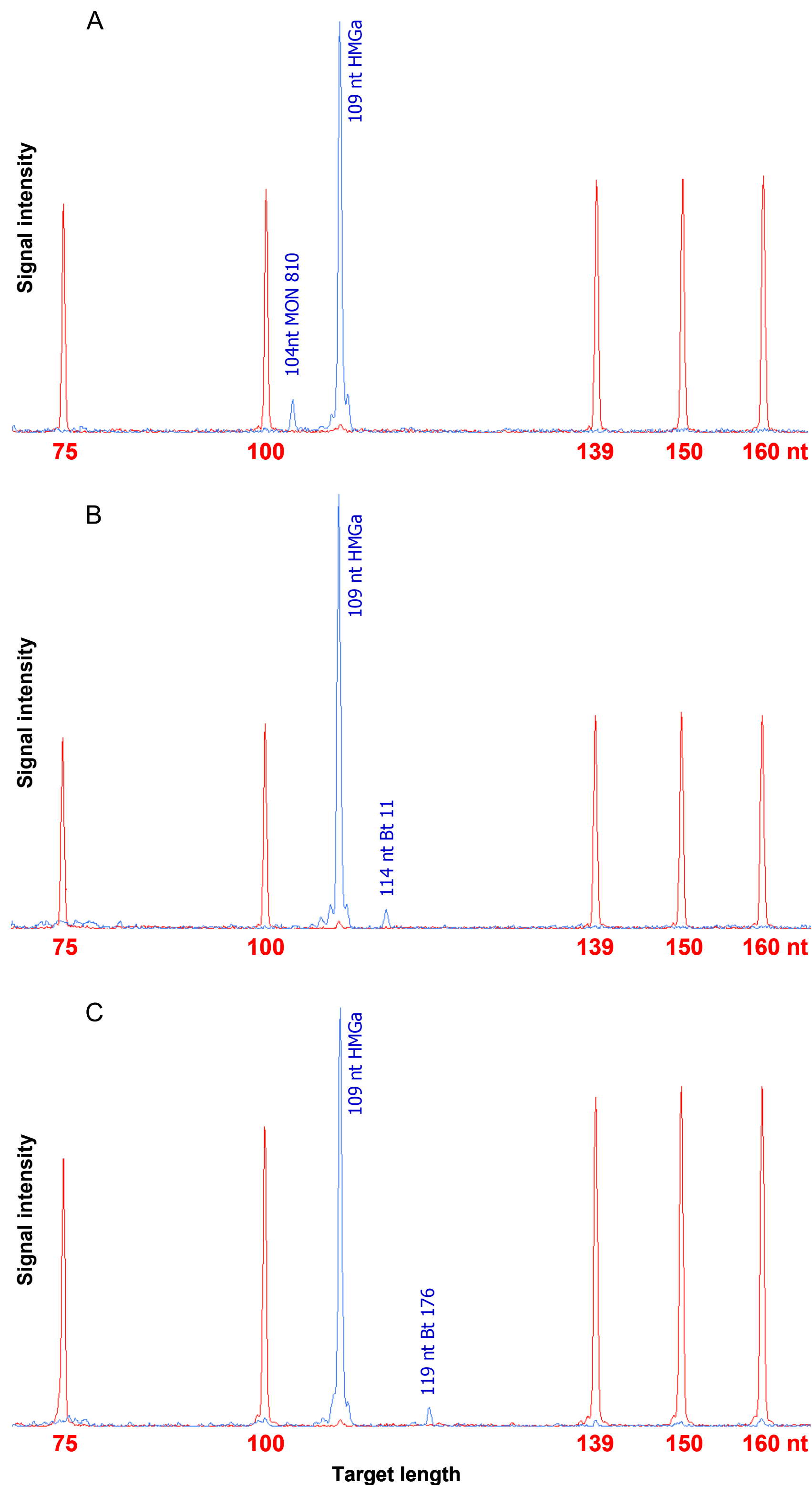

Figure 8 Assessment of the sensitivity of the LPA system for GM maize detection. (A) Electropherogram of a sample containing DNA of 0.1 % maize MON 810, (B) 0.1 % maize Bt 11, (C) 0.1 % maize Bt 176. Non-assigned signals correspond to the used size standard.

4.2.3 Construction of calibration curves for simultaneous quantification of GM maize lines

To demonstrate the suitability of this system for the simultaneous relative quantification of different GM maize lines, mixtures of adjusted DNA isolates from MON 810, Bt 11 and Bt 176 (with concentrations of 0.1 % to 5 % GMO) as well as negative controls free of transgenic material were analyzed. As shown in Figure 9, adequate signal intensities were obtained for all targets from 0.5 % to 5 % GMO content. Due to competitive amplification of all targets, the inversion of signal ratios was observable with increasing GMO concentration, thus indicating a correlation of signal intensity and GMO proportion.

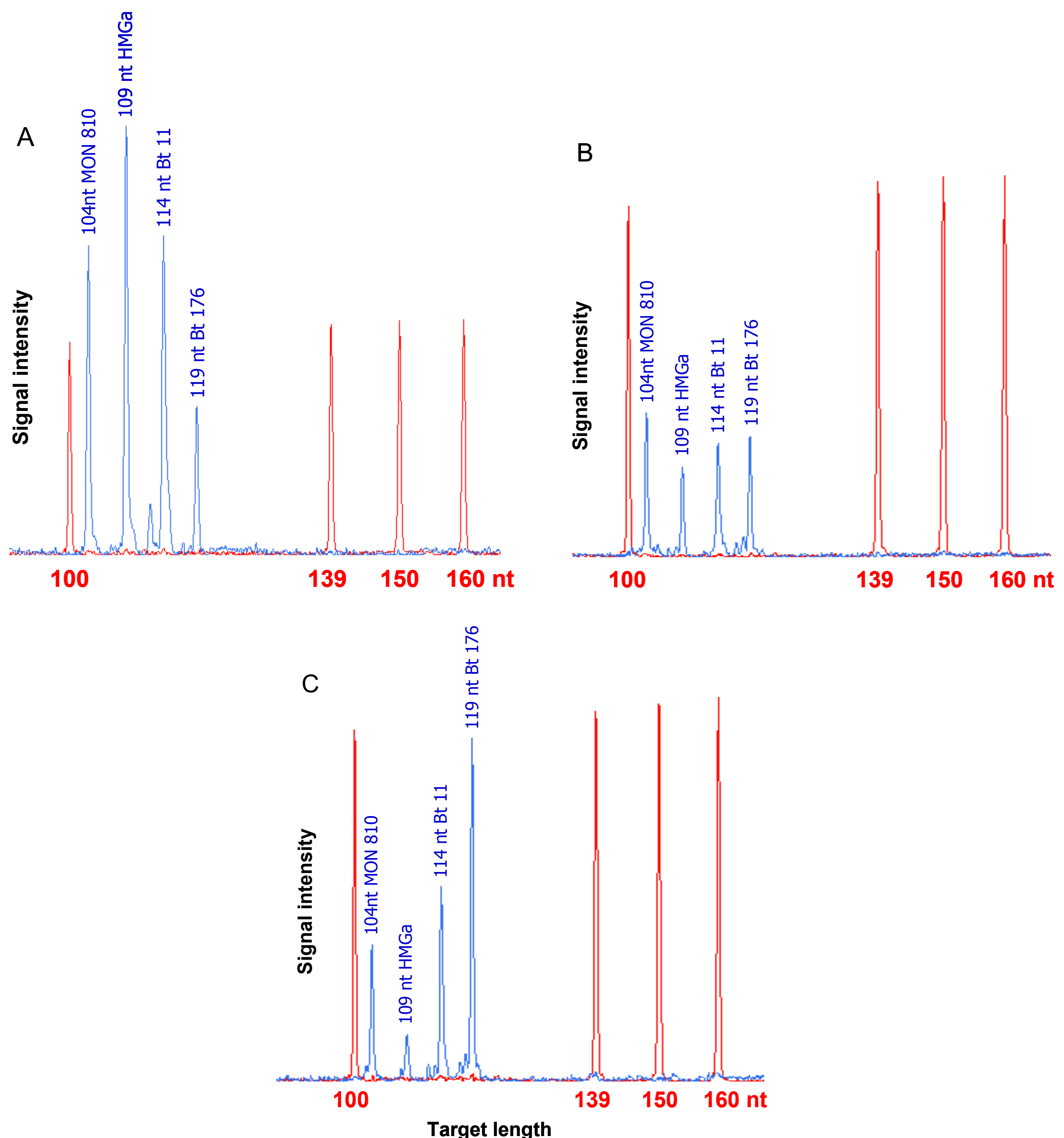

Figure 9 Range of the LPA system for GM maize quantification. (A) Electropherogram of a sample containing DNA of 0.5 % maize MON 810, Bt 11 and Bt 176 showing the three corresponding signals, (B) 2 % maize MON 810, Bt 11 and Bt 176. (C) Detection of 5 % maize MON 810, Bt 11 and Bt 176. Non-assigned signals correspond to the used size standard.

Signal intensities for quantitative fragment length analysis on an ABI 310 genetic analyzer have to be at least 100 extinction units to allow correct measurement of peak heights and areas, respectively; 50 units are sufficient for identification of peaks from background noise (Technical support Applied Biosystems, personal communication). This corresponds to an approximately 3-fold and 5-fold value of the background noise as it is used in chromatographic methods for evaluation of detection and quantification limits. Optimization of probe and competitor ratio yielded in quantifiable peak intensities of all samples containing maize MON 810 and Bt 176. As described in 4.2.2, the peak heights of samples containing 0.1 % of maize Bt 11 were in the range of 50 to 100 extinction units, which is insufficient for quantitative determination. Probably, the lower theoretical T_M of the Bt 11 probes affected the efficiency of hybridization performed at 60°C. Quantitative evaluation of Bt 11 was therefore restricted to the range of 0.5 % to 5 % GMO content. Future optimization to enhance the range of quantification will have to focus on optimum thermodynamic properties of the transgenic events and hybridization temperatures to guarantee sensitive approaches.
As illustrated in Figure 10, calibration curves were generated by determining the ratios between peak heights from the recombinant target and the reference gene, and plotting these against the actual GMO content of each standard. Data evaluation of the information obtained by electrophoresis was performed in Excel. Calibration curves showed a good linear correlation between the ratios of signal intensities and the increasing GMO content of the reference standards with $R^2 \geq 0.98$. In the case of maize Bt 11, the slope of the curve was nearly half as high as for the other targets, thus confirming the lower efficiency of the Bt 11 detection system. Experimental standard deviations of six-fold sample analysis were determined in a range of 17 – 53 % for all targets. These deviation values are high compared to applications using real-time PCR technology. They do not fulfill the minimum performance requirements for analytical methods of GMO testing by the ENGL and affected the performance of the approach for the analysis of maize MON 810 and Bt 176 [108]. Additional method optimization to enable the use of the LPA technique for the simultaneous relative quantification of multiple targets in one reaction focussed on the ligation reaction. Hybridization sites of lower melting temperature were chosen for the reference targets, the total volume of ligation reaction was increased to allow reproducible ligation reaction, and the ligation of probes was only performed once to generate an image of sample DNA content. However, the repeatability of signal intensities could not be enhanced and the standard deviations could not be reduced.
At present, the LPA technique allows the quantification of gene duplicates or triplicates – this is equivalent to differences of 50% or 33 % signal intensity, but it cannot be applied to

the relative quantification of trace amounts of genetically modified organisms. In future, experiments should focus on the optimization of competitive amplification using defined aliquots of the ligation products, thus increasing the reproducibility of the reaction. Furthermore, the influence of the melting temperature of hybridization sites on the sensitivity must be taken into account to ensure the sensitivity of the method.

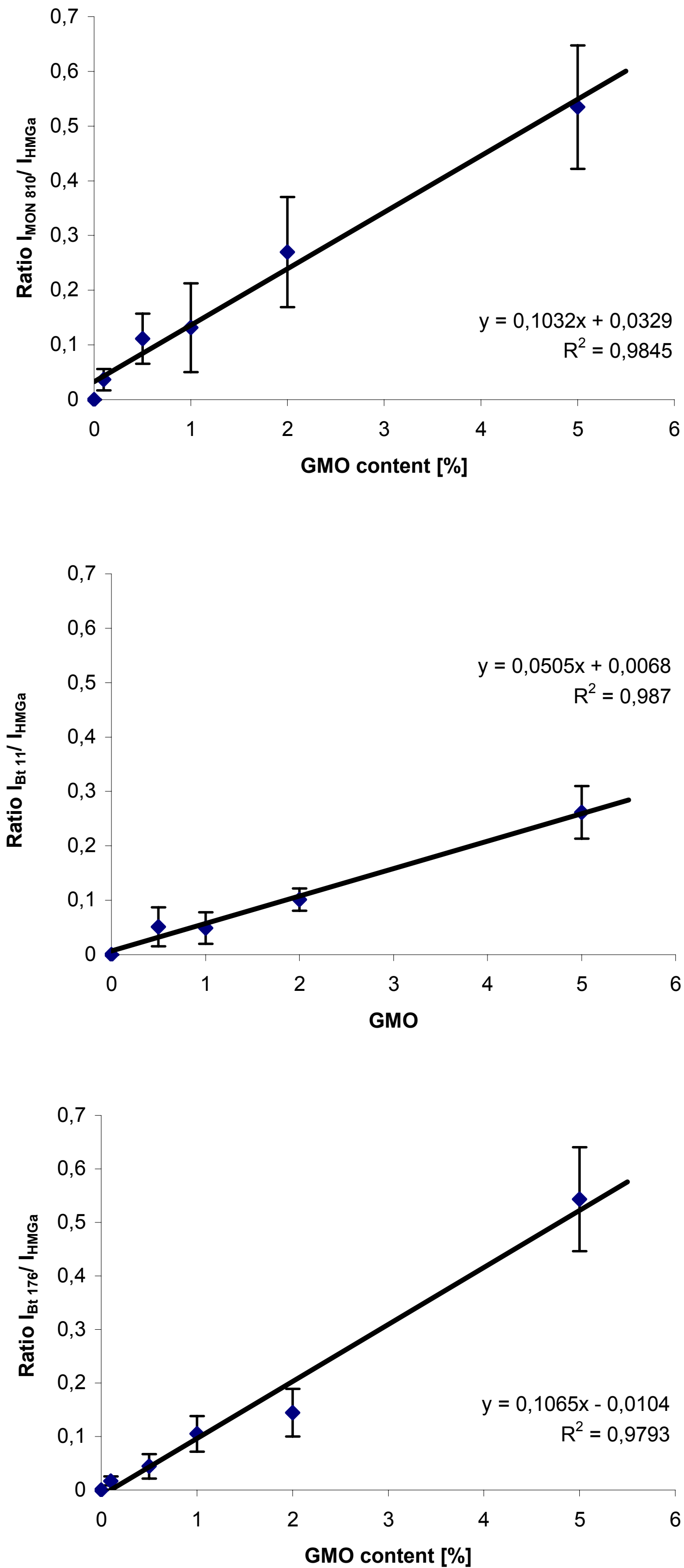

Figure 10 Calibration curves showing the correlation between ratios of peak heights from recombinant maize lines and maize reference target and the GMO-content of the standard. (A) Maize MON 810 ($I_{MON\ 810}/I_{HMGa}$), (B) Maize Bt 11(I_{Bt11}/I_{HMGa}) and (C) Maize Bt 176(I_{Bt176}/I_{HMGa}).

4.2.4 Summary

The application of the novel strategy of ligation-dependent probe amplification to the simultaneous relative quantification of GMO was shown for different recombinant maize events. Synthetic probe pairs were designed for the detection of the transgenic maize lines MON 810, Bt 11 and Bt 176 and of the maize reference gene (*HMGa*). The specificity was shown for all targets using database query and analyzing DNA extracts of different plant materials. The sensitivity of the LPA system was determined by assessment of the limits of detection (LOD) and quantification (LOQ) on the basis of signal intensities obtained by electrophoresis and fluorescence detection. Samples of mixtures from reference material containing 0.1 % maize MON 810, Bt 11 and Bt 176 were all detected positive whereas only the signals MON 810 and Bt 176 could be quantified. Analysis of reference material with a GMO proportion of 0.5 % of Bt 11 resulted in quantifiable peak data. Hence, the LOQ of Bt 11 is expected to be between the two standards of 0.1 and 0.5 % GMO content. Calibration curves showed good linear correlation between the relative signal intensities and the actual GMO proportion of the samples. Due to poor thermodynamic properties of the hybridization probes for Bt 11 detection, the range of quantification was limited to 0.5 % to 5 %. The restriction of the linear dynamic range of relative quantification to one order of magnitude is a disadvantage of the method in its present state. Furthermore, the relative quantification of traces of GMO by LPA provides comparatively high standard deviations that did not allow reproducible quantification. Nevertheless, the LPA technique presents a useful analytical strategy for sensitive and simultaneous determination of transgenic food ingredients from different sources with reduced efforts.

4.3 LPA Allergens

Food allergies are an important health issue in industrialized countries affecting 1-2 % of adults and up to 8 % of children and adolescents [100]. The increasing occurrence of food allergies and the possible health risk of unaware consumption of allergenic ingredients yielded in improved labeling regulations by the European Union. Recently, the new Directive 2003/89/EC and amendments establishes the labeling of several allergenic foods and products thereof by a list of ingredients with known allergenic potential. Currently, Annex IIIa of Directive 2007/68/EC comprises gluten-containing cereals, crustaceans, molluscs, fish, peanuts, soybeans, eggs, milk and dairy products (including lactose), nuts, celery, mustard, sesame seeds, lupine, sulfite and products thereof. To protect the health of consumers, the declaration of these ingredients has been made mandatory regardless of their amounts in the final product [7, 8, 107].

Appropriate detection methods are needed to assure the compliance of allergen labeling by surveillance authorities. Major challenges are the needs to check for the presence of food allergens at extremely low levels and to detect trace amounts of hidden allergens in composite and processed foods [9]. PCR-based methods amplifying specific DNA sequences offer alternative tools to the detection of allergenic of marker proteins for the species [10, 11]. In most cases, DNA presents a more stable analyte compared to proteins and is less affected by denaturation. In addition, species-specific sequences allow the discrimination of closely related organisms.

Suitable PCR assays for the detection and identification of individual food allergens have been developed some of the food allergens for which labeling is required [109-125]. At present, only a few Duplex-PCR systems are known allowing the simultaneous detection of peanut and hazelnut or wheat and barley at the same time [126, 127].

It can be expected that the number of food allergens that need to be labeled will be increasing. The aim of this study was therefore to develop and to validate a multitarget method for the simultaneous detection of allergens in different food matrices. The application of a ligation-dependent probe amplification (LPA) technique for the simultaneous detection of DNA from peanut, cashew, pecan nut, pistachio nut, hazelnut, sesame seeds, macadamia nut, almond, walnut and brazil nut in a single reaction is described. Ligation-dependent PCR developments and applications have been described for nucleic acid detection, medical diagnostics and event-specific detection and relative quantification of GMO [96, 97, 130-136]. The technique does not amplify the target sequences, but is rather based on the amplification of products resulting from the ligation of bipartite hybridization probes. The use of this analytical strategy results in a flexible system that can be complemented with further hybridization probes to broaden the range of target sequences to be detected. This approach has been realized by the development

of a modular system allowing the simultaneous detection of several GMO targets corresponding to different levels of specificity on a one-tube assay (cf 4.1) [143].

4.3.1 Design/ choice of target sequences

Different tree nuts (macadamia, cashew, pecan, walnut, brazil nut, pistachio, almond and hazelnut) as well as peanut and sesame were chosen as examples for the detection of allergenic components in foods by LPA. They are often contained as hidden allergens in pastries, candies or chocolate and present potential health risk for allergic persons. Details of the selected target sequences and the GenBank accession numbers of the LPA primers and probes are listed in Table 4. Concentrations of the LPA probes are shown in Table 6 (cf. 3.2.6). The probes mostly detect genes encoding for plant food proteins of the cupin and prolamin superfamily, respectively, which are known to cause IgE-mediated allergic reactions. The simultaneous detection of a DNA mixture (20 ng/µL) containing all targets equally is shown in Figure 11.

The primer sequences described by Schouten et al. [96] for MLPA analysis were also used in this study. They were tested regarding their suitability for analyzing foods by database enquiry via NCBI GenBank and PCR using DNA of different plant species. (cf. 4.1.2). The use of spacer sequences between PBS and hybridization sites rendered ligation products with lengths characteristic for each of the target DNA. Differences of 4 nt in length have been shown sufficient for unequivocal determination of the amplification products using POP-6™ polymer (cf.4.1.2). Again, simple repeats of GT bases were used for the spacer sequences to avoid intra- and intermolecular hybridizations.

Using this approach, all target signals were sufficiently separated. The signal obtained for pistachio nut overlapped with one of the standard signals (100 nt); however, owing to the use of different fluorescent dyes for standard and target probes a discrimination of the two signals is possible.

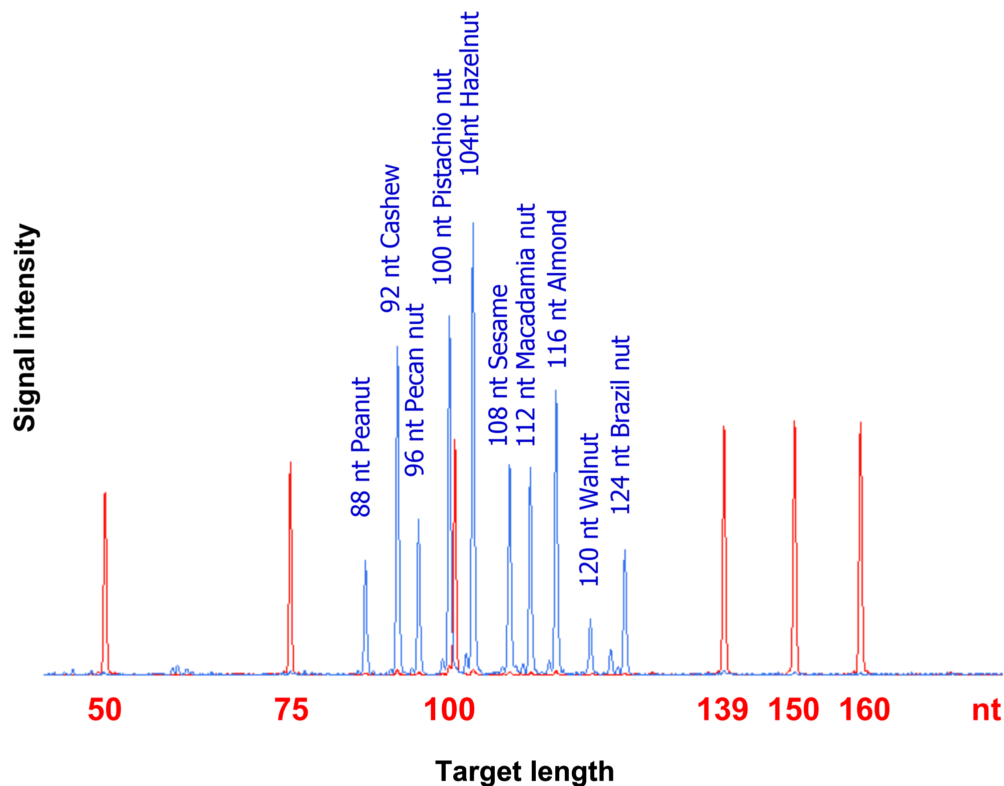

Figure 11 Scope of the LPA system to detect allergenic food ingredients. Electropherogram of a sample containing a DNA mixture (20 ng/µL) of peanut, cashew, pecan nut, pistachio nut, hazelnut, sesame, macadamia nut, almond, walnut and brazil nut.

4.3.2 Evaluation of target specificity

To avoid cross homologies the specificities of the probe target sequences were first evaluated by BLAST search within NCBI GenBank by Beacon Designer software. Additionally, each probe was checked with DNA extracted from the other target species. The performance of the LPA system was examined using DNA extracts of the ten targets adjusted to 20 ng/µL and different mixtures prepared to simulate composed food products with different nut proportions. No unspecific signals were observed for almond, peanut, pecan, pistachio, hazelnut, sesame, macadamia, walnut and brazil nut probes. Furthermore, the differentiation between the phylogenetically closely related tree nuts pecan and walnut could be achieved. Published sequencing data were used to design the ligation probes with only four different DNA bases [121, 124]. Cross-reactivity of the cashew probes with other members of the Anacardiaceae family, specifically pistachio and mango, could be eliminated by detailed characterization of the target and a new design of the probes. Different pairs of primer were designed to amplify the target region of the 2s albumin gene (ana o3 allergen). PCR analysis using one of the primer pairs and DNA from cashew, mango and pistachio resulted in specific amplification of a 103 bp DNA

section of cashew DNA [144]. No signals were observed when DNA of pistachio or mango were used in the PCR reaction (data not shown). The region that was also used for specific detection of cashew DNA by real-time PCR analysis (cf. 4.4.1) [144] was therefore chosen as hybridization site for the cashew LPA probes.

Subsequently, the specificity of the method was tested by analyzing DNA from organisms related to the selected targets and from other organisms that are to be expected as ingredients of composed foods. Non-coding regions of chloroplast DNA were amplified for verification [145]. The species tested with all LPA probes are listed in Table 10. No false positive signals were observed for peanut, cashew, pecan, pistachio, hazelnut, sesame, macadamia, walnut and brazil nut. The probes developed for the detection of almond DNA also yielded positive results in the presence of DNA from apricot, nectarine, peach and plum due to their phylogenetical relation. Further characterization of the target by sequencing will be necessary to increase the specificity of the almond detection.

Slight peaks at n-1 and n-2 nucleotides are caused by impurities of the synthesized oligonucleotides that could be reduced by a new synthesis but could not be eliminated completely. As shown exemplarily for walnut in Figure 9, especially longer probes excite clear signals of shorter amplicons. Resynthesis using further purification by the manufacturer (Biolegio B.V., Nijmegen, The Netherlands) significantly reduced the unrequested signals. Minor effects discussed above (4.1.2) like slippage and nucleotide addition of the polymerase can be induced by the enzyme itself.

Table 10 Species used for the determination of the target specificity of the LPA method for allergen detection

plants			**animals**
anise	garlic	plum (leaf)	beef
apple	ginger	pumkin seed	chicken
apricot (leaf)	lemon grass	raspberry	duck
banana (leaf)	linseed	(leaf and fruit)	turkey
basil	maize	rice	pork
blackberry (leaf)	mango	rye	
cardamom seeds	(pulp and paring)	sour cherry	**microorganism**
carrot	nectarine (leaf)	soya	
cinnamon	nutmeg	strawberry	
coconut	oregano	(leaf and fruit)	yeast
coriander	parsley	sunflower seed	
cumin	peach	sultana	
curcuma	pear	sweet cherry	
currant	pepper (black)	wheat	
fennel	pimento		
	pine nut		

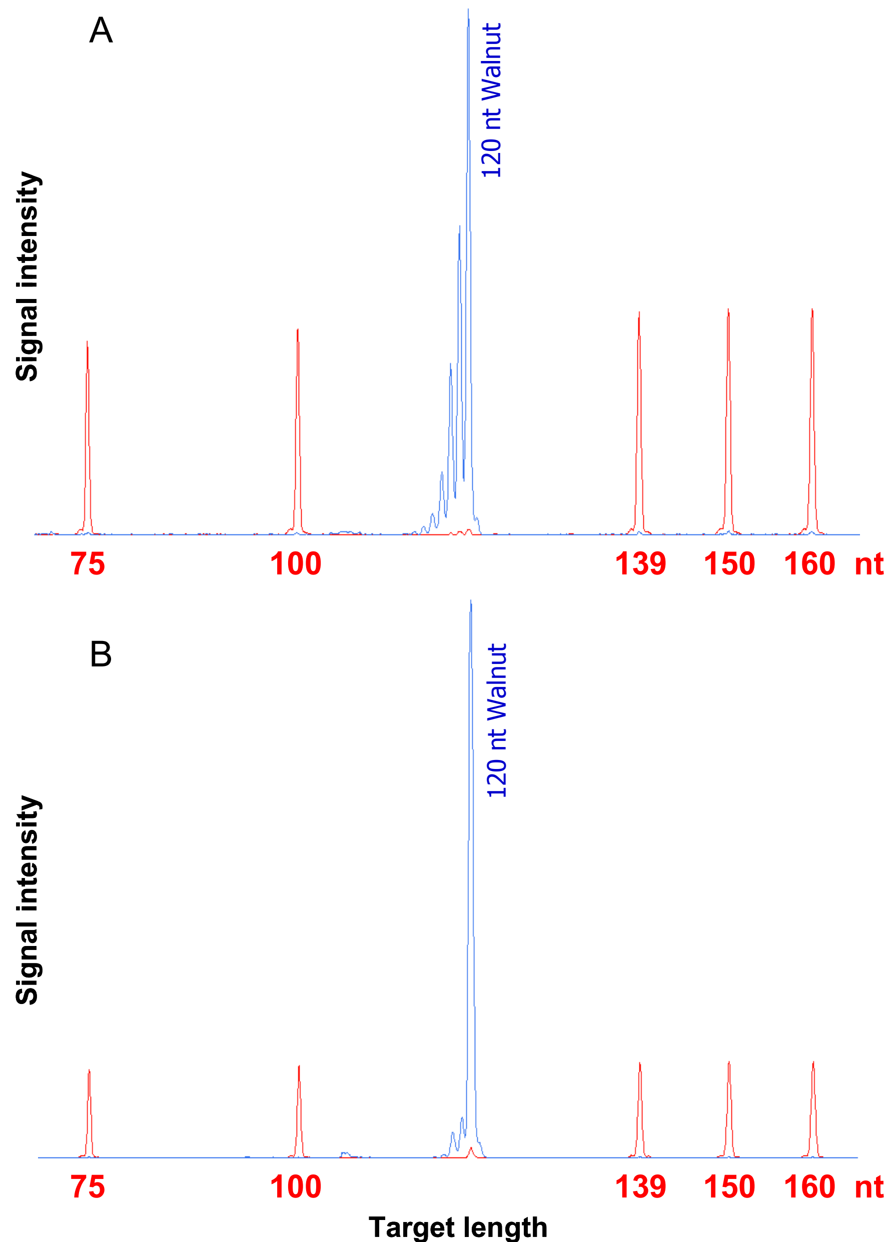

Figure 12 Improved purity of long probe oligonucleotides (A) Electropherogram of the walnut LPA amplicon. (B) Electropherogram of the purified probes using the same sample DNA.

The fragment lengths determined for each target were highly reproducible (SD ± 0.2 nt). The observed slight shifts (≤ 1 nt) of the absolute length value (cf. Table 11) are well-known effects arising from differences in the mobility of length standards and of the analyzed fragments caused by different labelling dyes and by differences in the structure and base compositions of the DNA [137, 138].

Table 11 Amplicon length and retention time of LPA Probes for the detection of allergens

Target	Amplicon length [nt]	Retention time [nt]
Peanut *Arachis hypogaea*	88 bp	87,10 ± 0,13
Cashew *Anacardium occidentale*	92 bp	91,16 ± 0,17
Pecan nut *Carya illinoinensis*	96 bp	94,67 ± 0,17
Pistachio nut *Pistacia vera*	100 bp	99,37 ± 0,18
Hazelnut *Corylus avellana*	104 bp	102,75 ± 0,12
Sesame seeds *Sesamum indicum*	108 bp	108,11 ± 0,13
Macadamia nut *Macadamia integrifolia*	112 bp	111,07 ± 0,14
Almond *Prunus dulcis/* *Amygdalus communis*	116 bp	114,84 ± 0,12
Walnut *Juglans regia*	120 bp	119,80 ± 0,11
Brazil nut *Bertholletia excelsa*	124 bp	124,84 ± 0,09

4.3.3 Assessment of the sensitivity of the LPA system for the detection of allergens

Due to the lack of appropriate reference materials for the detection of allergens, different food matrices in which the selected plants typically occur were chosen to evaluate the sensitivity of the LPA system. Chocolate was spiked with peanuts and hazelnuts, pesto with cashew nuts, and cookies with peanuts. In addition, walnut cookies spiked with peanuts, hazelnuts, pecan and macadamia nuts were self-prepared. These examples were selected to simulate fraudulent labeling or the unintended contamination of foods with different nuts. Starting materials and ingredients were checked for the absence of any target of the LPA system.

Two independent DNA extractions of the spiked samples were analyzed in duplicate by LPA to determine the limits of detection. Fragment length analysis was performed with the pure and 1:20 diluted PCR product. The LOD was assessed as the least concentration for which all results were consistently positive. For peanut, hazelnut and cashew nut, limits of detection of 5 mg/kg were determined in the matrices chocolate, cookie and pesto, respectively.

To study the influence of unequal proportions of allergenic components in a food, i.e. high excess of one of the LPA targets in the sample, on the sensitivity of the method, cookies containing 25% (250,000 mg/kg) walnuts were spiked with defined amounts (1 – 10,000 mg/kg) of four other nuts. Due to competitive amplification of the probes during PCR, under these conditions the added reagents are mainly used to amplify the excess of walnut ligation products. Consequently, the sensitivity for the nuts contained as trace amounts is reduced, resulting in LODs of 1000 mg/kg for peanut, hazelnut, pecan, macadamia and 100 mg/kg for hazelnut, respectively (Table 12).
This inherent feature of the multi-target LPA method has to be taken into account, when traces of targets are to be simultaneously detected in the presence of a high amount of one of the other LPA targets.

Table 12 Limits of detection for the detection of allergens by LPA, real-time PCR and ELISA

Sample	**Detection limit [mg/kg]**		
	LPA	**Real-time PCR**	**ELISA**
Peanut chocolate	5	5	5
Hazelnut chocolate	5	10	10
Peanut-spiked cookie	5	0.5	5
Pesto cashew	5	2	*
Walnut cookies with peanut, hazelnut, pecan, macadamia	1000 (peanut, pecan, macadamia) 100 (hazelnut)	1 (peanut) 10 (hazelnut)	100 (peanut) 1 (hazelnut)

* no method available

4.3.3.1 Comparison LPA – ELISA – Real-time PCR

The samples used to determine the limits of detection of the LPA method were also analyzed by real-time PCR and ELISA (Table 12). For the samples analyzed by the commercially available ELISA kits, the LODs determined for hazelnut and peanut were slightly higher that those given by the manufacturer. For the LODs determined for these targets by the real-time PCR methods, such a comparison was not possible because the information given by the manufacturer refers to copies of genomic DNA. The limits of detection observed for peanuts, hazelnuts and cashew nuts in chocolate and pesto by LPA were similar to those determined for the tested ELISA and real-time PCR approaches. The real-time PCR method applied for the detection of peanuts showed higher sensitivity; however, the application of this kit also resulted in positive signals for the non-spiked

walnut reference cookie. Therefore, the reliability of these results remains questionable. The hazelnut ELISA test also showed cross reactivity to walnut (0.001 – 0.036 %); owing to the false-positive results a detection of hazelnut traces in the presence of walnut is not possible with this kit [146, 147].

4.3.4 Analysis of retail samples

A variety of commercial foods were tested for the presence of the LPA targets. A total of 39 samples from different food categories were analyzed. All DNA extracts were checked for PCR inhibition and amplifiability of DNA prior to LPA analysis by amplification of non-coding regions of chloroplast DNA (cf.4.3.2) [145]. Results obtained for spreads, sausages, dressings, dairy products and sweets are exemplarily shown in Table 13.

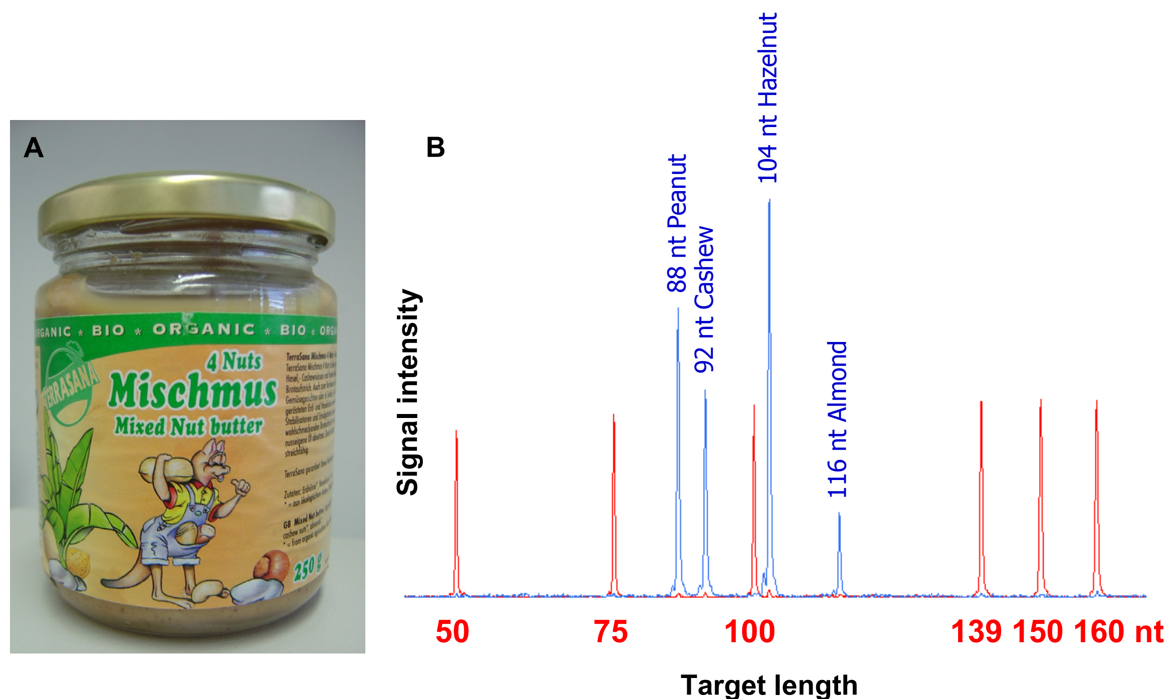

Figure 13 Analysis of a sample organic mixed nut butter. (A) Sample label. (B) Electropherogram showing the detection of for tree nuts contained in the sample. Non-assigned signals correspond to the used length standards.

The results obtained for the samples “organic mixed nut butter” and “gingerbread” demonstrate the advantages of the developed multi-target LPA method. In both cases the simultaneous detection of the allergenic ingredients declared on the label could be achieved. Figure 13 shows the results obtained for the sample “organic mixed nut butter”.

Except for walnuts in a Thuringian sausage, the presence of ingredients that had been declared on the label could be confirmed by LPA analysis in all samples. In addition, information given as part of a precautionary labeling in some of the retail samples could be confirmed or specified. Considering the described reduction in sensitivity for targets

contained as trace amounts in the presence of a high amount of one of the other LPA targets, the detection of undeclared allergenic ingredients may be even further improved by analyzing samples with probe mixes that exclude the probes that would amplify the quantitatively dominating and declared allergenic ingredients.

A still existing limitation of the method is the missing differentiation of almond from apricot, nectarine, peach and plum. This became obvious for the sample “gingerbread”: The signal detected for almond could not be assigned unequivocally to almond, because apricot kernels had also been declared in the list of ingredients.

Quantitative conclusions on the contaminations found in the retail samples are difficult, but concentrations at the lower ppm range are likely.

Table 13 Analysis of commercially available samples by LPA

Product	Declaration/ Ingredients	Product liability	Detection
Spreads			
organic mixed nut butter	peanuts, hazelnuts, cashews, almonds	-	peanut, cashew, hazelnut, almond
Nutella	hazelnut 13 %	-	hazelnut
Sausages			
original Thuringian sausage	walnuts	-	n.d.
mortadella with pistachios	pistachios 551%	-	pistachio
Convenience food dressings			
pesto alla Genovese	cashew nuts	-	cashew
saté dressing	peanuts 21%, peanut flavor	-	peanut
pesto goutweed (Aegopodium podagraria)	macadamia nut	-	macadamia nut
Dairy products			
yoghurt with almonds	almonds 2%	-	almond
milkshake pistachio-cocos	pistachio pulp	-	pistachio
Sweets/ Cookies			
chocolate bar	hazelnut 5 %	traces of almond, peanut and other nuts	hazelnut
hazelnut bar	hazelnut mark 3,4%	traces of nuts and other seeds	hazelnut
hazelnut bar with honey	hazelnut 66 %	traces of peanut, sesame or other nuts	hazelnut, peanut
peanut bar with honey	peanut 68 %	traces of sesame or nuts	peanut, hazelnut
sesame seed bar with honey	sesame 68 %	traces of peanut or other nuts	sesame
praline hazelnut	hazelnut 30,5 %, almonds	traces pistachio	hazelnut, almond
praline pistachio	almonds 12,5 %, pistachio 6,5 %	traces hazelnut	almond, pistachio
praline coconut	almonds 5 %	traces egg, hazelnut, pistachio	almond, pistachio

4.3.5 Summary

The LPA system for the detection of food allergens was shown to be a specific and sensitive detection method suitable for the simultaneous detection of peanut, cashew, pecan nut, pistachio nut, hazelnut, sesame seeds, macadamia nut, walnut and brazil nut in the lower ppm (mg/kg) range. Synthetic oligonucleotides as hybridization probes for all ten targets were designed.

The specificity of the system was demonstrated by analysis of DNA from more than 50 different plant and animal species. However, the specificities of the probes targeting almond DNA have to be increased in order to be able to differentiate almond from the phylogenetically related species apricot, nectarine, peach and plum. Further characterization of the almond target, e.g. sequencing as applied for the cashew target, will be necessary.

The limit of detection of the method of single allergens in different food matrices was determined to 5 mg/kg. Comparable results could be achieved using real-time PCR and ELISA. This proves the suitability of the LPA method to detect traces of allergens in different food matrices. More information about individual limits of detection of the LPA method in real food products is necessary. The lack of certified reference materials in the field of allergen detection makes development and validation of appropriate methods more difficult. Further reference materials with certified concentrations of analyte are of particular importance to test the applicability of the method to all target species.

The important application to manifold food matrices was shown by analysis of retail samples of various product groups. Animal foods like sausage and dairy products could be examined as well as vegetable dressings and spreads. No problems arise from difficult PCR inhibiting matrices like chocolate and other sweets. Nevertheless, the implementation of an additional internal amplification control would be helpful to avoid false negative signals due to PCR inhibition and to assure amplifiability of sample DNA. Thus, the time-consuming conventional PCR targeting a non-coding region of chloroplast DNA and detection by ethidium bromide stained agarose gelelectrophoresis could be circumvented. Integration of a synthetic target and probes as used for the LPA GMO system would be conceivable.

The application of this novel analytical strategy represents a useful tool in order to assure the surveillance of the established legislation and for food industry to comply with the regulations for allergens in the European Union. The modular system allows the extension to further target sequences of interest.

4.4 Detection of cashew nut by a specific real-time PCR method

Tree nuts (e.g. hazelnut, walnut, Brazil nut, and cashew) are among the most common food allergens worldwide and therefore, labeling according to the Annex IIIa of Directive 2003/89/EC has been made mandatory since November 2005. Allergy to cashew nuts is the second most commonly reported tree nut allergy in the USA [149]. Consumption of cashew nuts as snack or ingredient in various foods is popular not only in North America and associated allergies are reported globally [150, 151]. Cross-reactivities are described for patients sensitized to pistachio which belongs to the same botanical family [152].

Appropriate analytical methods are necessary to allow specific and sensitive detection of cashew and to assure the surveillance of labeling requirements. Mainly two techniques, protein-based immunoassays and DNA-based polymerase chain reaction are currently used for analysis of allergenic foods. At present, one sandwich ELISA for the detection of cashew nut is described in literature [151]. This method was developed to detect the specific cashew major protein (CMP or anacardein) at a minimum quantity of 1 mg/kg in food. However, reported cross reactivities to pecan, walnut, pistachio and sunflower seeds demonstrate limitations of this assay. In addition, one PCR system has been published for the detection of cashew nut DNA using real-time PCR [109]. The sensitivity of the method was determined to be 100 mg/kg in spiked chocolate cookies, which represents a relatively high level of hidden allergens or cross contaminations in foods especially for tree nut-sensitive persons. There is general agreement that methods suitable for the detection of trace amounts of allergenic components should be sensitive down to 1 mg/kg [9-11]. Testing of the specificity was limited to four further tree nuts and peanut, whereas other edible members of the Anacardiaceae family were not considered.

The objective of this study was therefore to develop a specific real-time PCR method allowing the sensitive detection of cashew nut in different food matrices. The assay design was focussed on the specific differentiation from phylogenetically related species and other allergenic cross-reactive tree nuts. The experiments performed in the following study concentrated on extensive in-house method validation in accordance to accepted criteria for method development for GMO analysis [108].

4.4.1 Design of a real-time PCR method for the specific detection of cashew nuts

Different allergenic cashew proteins of the vicilin seed storage protein family (Ana o1), the legumin family (Ana o2), and the 2S albumin family (Ana o3) have been identified and characterized previously [128, 150, 153-154]. The corresponding coding nucleotide sequences were analyzed by database enquiry via NCBI GenBank and BLAST search. No similarities to homologous sequences of other nuts or closely related species were

found within the search basis for GenBank accession no AY081853 coding the Ana o3 cDNA. Therefore, the design of primers and probe was based on this target using the software mentioned above (c.f. 3.1.4.4). The primer pair Cashew F and Cashew R amplifies a 529 bp long fragment that was used for cycle sequencing verifying the sequence identity of the cashew reference DNA, for generating DNA standards and dilution series, and as spike-DNA for inhibition control reactions. Specific primers Cashew RT F and Cashew RT R as well as the Cashew RT TaqMan™ probe allow real-time PCR detection of a 103 bp DNA section of the gene encoding the Ana o3 cashew nut allergen (Figure 14).

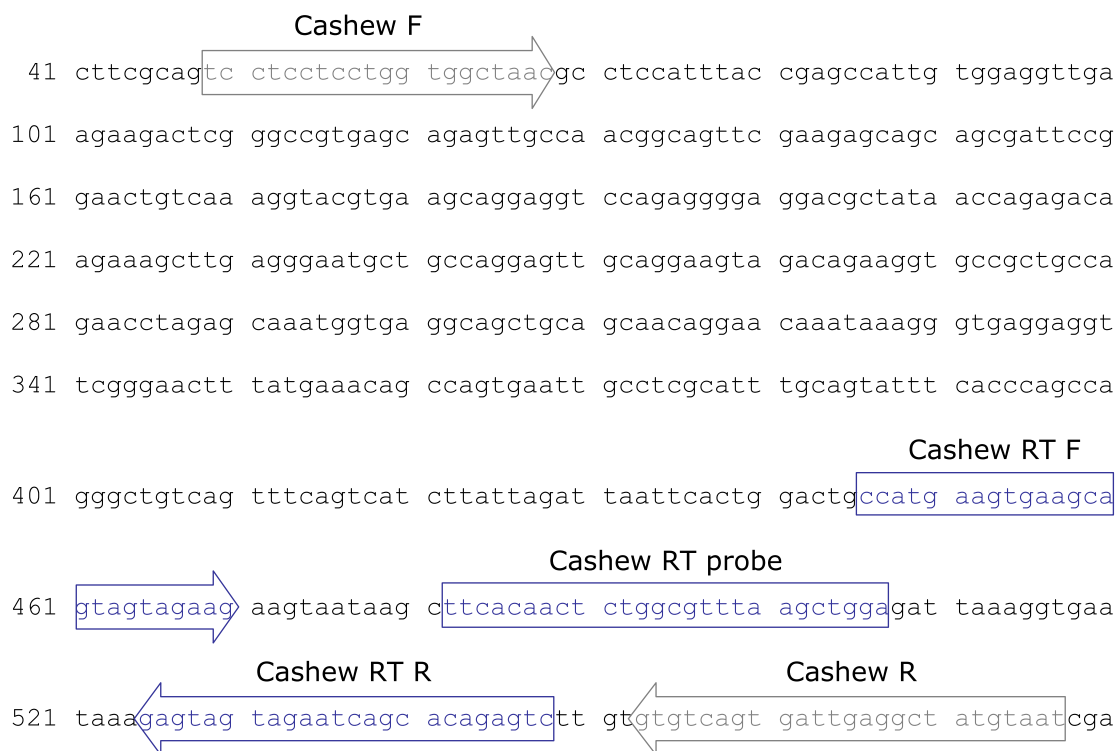

Figure 14 Targeted region of primers and probe localized on the gene encoding the Ana o3 cashew nut allergen (GenBank accession no AY081853)

4.4.2 Assessment of the specificity

The lack of homologies of the selected oligonucleotide sequences was demonstrated by database enquiry within the NCBI GenBank by Beacon Designer software. Additionally, both primer pairs were tested with DNA extracted from other edible members of the Anacardiaceae family, i.e. mango and pistachio as well as further allergenic organisms and tree nuts namely sesame, peanut, hazelnut, macadamia, almond, Brazil nut, walnut, and pecan. No unspecific amplification signals were observed using the conventional primer pair or the real-time PCR system.

Subsequently, amplifiable DNA of organisms that are concurrently found in composed foods was used for evaluation of the specificity. To eliminate false negative results due to PCR inhibition the absence of inhibitors in the DNA extracts was verified by amplification of noncoding regions of chloroplast DNA [142]. In detail, the following plant species were tested: anise, apple, apricot, banana, basil, pear, blackberry, curcuma, strawberry, fennel, yeast, sweet cherry, raspberry, ginger, cacao, cardamom seeds, carrot, garlic, coconut, coriander, currant, cumin, pumpkin seed, linseed, maize, nutmeg, nectarine, oregano, parsley, black pepper, peach, pimento, pine nut, rice, rye, sour cherry, soya, sunflower seed, sultana, wheat, cinnamon, lemon grass and plum. In total 50 different species yielded negative results with the real-time PCR system, thus confirming the high specificity of the developed detection method.

In addition, the pesto matrix used for spiking experiments designed to test the applicability of the method and to determine the LOD in real food matrices was successfully checked for the absence of cashew DNA prior to spiking.

In contrast, another real-time PCR method for the detection of cashew nut published previously [109] was tested with other members of the Anacardiaceae family especially mango and pistachio. Positive signals were detected using DNA extracted from pistachio, therefore the specificity of the method is limited and not suitable for the differentiation of allergenic food components from cashew and pistachio.

4.4.3 Assessment of the sensitivity

Genomic DNA extracted from cashew nuts was used for the evaluation of the sensitivity of the real-time PCR system. Since no data on the molecular weight of a single haploid genome of cashew were available, DNA concentrations were determined fluorimetrically and dilution series of genomic DNA were prepared ranging from 0.1 pg/µl to 20 ng/µl. In addition, PCR products of the 529 bp cashew specific sequence were generated by primers Cashew F and R and used as standards. Conversion of the measured concentrations to copies per µl was done according to the formula given in Figure 15, which is based on the average molecular weight per bp of 660 g/mol, the length of the amplicon and the Avogadro's number. Serial dilutions of the PCR product ranging from 1 to 10^7 copies per reaction were prepared for analysis of sensitivity.

$$c(\text{copies}/\mu l) = \frac{c(ng/\mu l) \cdot 6{,}022 \cdot 10^{23} \cdot mol^{-1}}{660 \cdot 10^{9} ng \cdot mol^{-1} \cdot bp^{-1} \cdot 529 bp}$$

Figure 15 Calculation of copy numbers/µl from DNA concentration

The limit of detection as the lowest amount of template that could be reliably detected was assessed to be the least dilution step where all five replicates of the dilution series were detected positive. Hence, the absolute detection limit of the real-time PCR method was determined to be 10 initial template copies of the amplicon and 0.5 pg genomic DNA, respectively. These absolute values obtained by analysis of highly purified PCR products and pure genomic cashew DNA are rarely applicable to real food samples. Therefore, practical or functional limits of detection should be determined by analysis of real food matrices (cf. limit of detection in matrices) as described for GMO analysis [108].

Calibration curves of genomic DNA and PCR products were generated by plotting the mean cycle threshold (C_T) values of five replicates per dilution step against the logarithm of the corresponding DNA amount and copy number, respectively. As shown in Figure 16, the obtained regression lines had a slope of -3.4 and -3.3 respectively and a correlation coefficient higher than $R^2 > 0.99$ for both. The amplification efficiencies calculated from the slopes range between 97 and 101 percent, thus minimum acceptance/ performance criteria as defined for GMO analysis by the ENGL are fulfilled by the developed real-time PCR method [108].

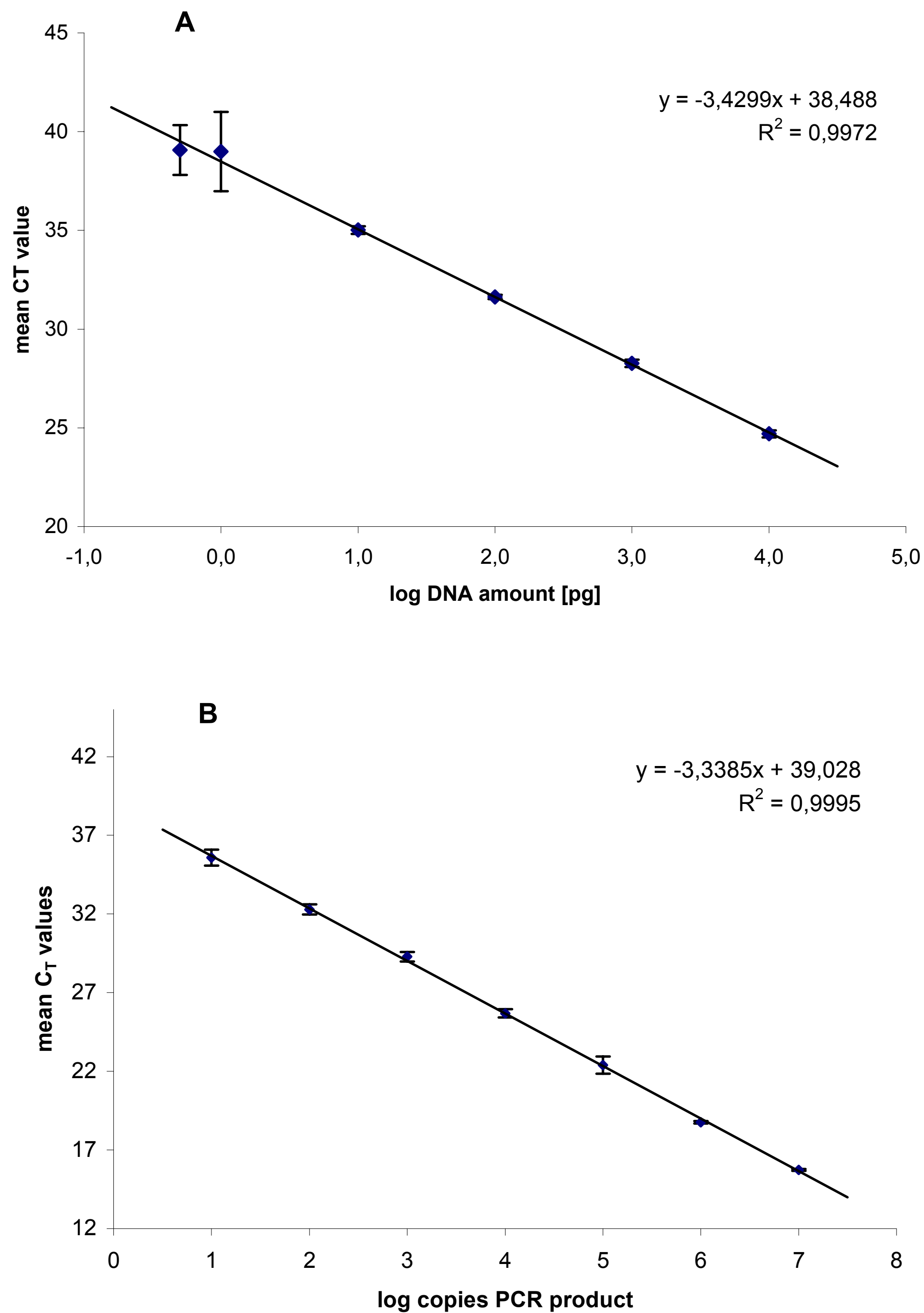

Figure 16 Standard curve of serial dilutions of (A) genomic cashew DNA in the range of 0.5 to 10^4 pg, (B) cashew PCR amplicons in the range of 1 to 10^7 copies.

Furthermore, DNA isolated from cashew free pesto was spiked with 10 copies per reaction of the cashew PCR amplicon and analyzed in 20 replicates. The false negative rate was determined to be 0 % with a mean C_T value of 37.8 ± 1.1, so even 10 copies present in the DNA extracted from the food matrix were clearly detectable by the method.

4.4.4 Assessment of the robustness

To determine the robustness of the method, the experiment was varied by transferring the optimized PCR protocol to another real-time PCR cycler, simulating influences of small assay modifications/deviations as described by the ENGL method performance requirements used for GMO analysis [108]. Real-time PCR analysis of 10 replicates was performed on the ABI 7900 HT and the Stratagene Mx 3000P instrument starting with 100 copies of the cashew specific target per reaction. The mean C_T values were determined to 32.8 ± 0.2 for the run on the ABI 7900 HT and 33.1 ± 0.2 using the Stratagene Mx 3000P cycler. Comparable results were obtained on both instruments with an only slight shift of the mean C_T value and no differences in standard deviation. The calculated relative standard deviations (CV) of < 1 % for both experiments proved that small variations of the assay did not influence the performance of the method.

Table 14 Comparison of C_T values obtained by real-time PCR analysis on the ABI 7900HT and on the Stratagene Mx3000P instrument

Replicate	ABI 7900 HT	Stratagene Mx3000P
1	32.55	33.32
2	32.74	33.03
3	32.91	33.07
4	32.96	32.61
5	33.08	33.31
6	32.68	33.34
7	32.86	33.15
8	32.71	33.22
9	32.66	33.00
10	32.35	33.44
Mean C_T	32.75	33.14
SD	0.21	0.24
CV [%]	0.64	0.72

4.4.5 Limits of detection in matrices – Applicability

As an important step in the method validation process the application of the method to real food matrices should be assessed. In this regard, pesto was chosen as a common food matrix, where cashew nuts are frequently present either as ingredient or as carry-over contamination by other nuts. Its use is widespread as dressing with many variations of herbes, spices and vegetables like calabrese with paprika/red pepper, siciliana with tomatoes, genovese with basil, rocket and bear's garlic. The challenge of inhibitory effects of the herbs and spices contained in pesto required an effective DNA extraction procedure and optimal assay conditions. Due to the lack of appropriate reference materials for detection of allergens the most common and frequently used basil pesto (previously tested for the absence of cashew DNA, c.f. 4.4.2) was selected and spiked with ground cashew nuts. Stepwise dilutions of the spiked pesto with cashew-free pesto were done down to 1 mg/kg. The practical LOD by real-time PCR analysis of the spiked samples was determined to be 2 mg/kg cashew nut in the matrix basil pesto. Analysis of four PCR replicates of samples containing 10, 5 and 2 mg/kg cashew resulted in reproducible mean C_T values of 36.2 ± 0.2, 36.3 ± 0.3 and 38.1 ± 0.4 (mean of three values), respectively. No positive signals were observed for samples containing 1 mg/kg cashew nut.

4.4.6 Analysis of retail samples

A variety of commercial foods was tested for the presence of cashew DNA with the validated real-time PCR method. In addition to the cashew-specific real-time PCR reaction, the DNA extracts were spiked with 100 copies of the cashew target DNA in separate reactions in order to control potential PCR inhibition by the sample matrix. If necessary suitable dilution of the sample extracts was done to avoid false negative PCR results due to PCR inhibition.

A total of 17 food samples of different product groups were analyzed. In Table 13 the results of the examined foods mainly pestos, but also dairy products, sweets and spreads are shown. All cashew nut ingredients declared on the label could be detected by the real-time PCR method. The absence of cashew was confirmed for samples that did not indicate cashew in the list of ingredients or as part of an advisory labeling such as "may contain…".

The successful determination of cashew proportions in all commercial samples demonstrates the suitability of the developed real-time PCR system for the analysis of different food matrices in addition to the pesto matrix.

Table 15 Detection of cashew nut DNA in commercially available samples

Product	Declaration/ Ingredients	Detection of Cashew
Pestos		
Pesto verde 1	Pine nuts	-
Pesto verde 2	Cashew nuts, pine nuts	+
Pesto alla Calabrese	-	-
Pesto alla Siciliana	Cashew nuts, walnuts	+
Pesto alla Genovese 1	Cashew nuts, pine nuts	+
Pesto paprika	Cashew nuts, almonds, pine nuts	+
Pesto alla Genovese 2	Cashew nuts	+
Pesto basil	Pine nuts, walnuts	-
Pesto rocket	Almonds, cashew nuts, pine nuts	+
Pesto bear's garlic	-	-
Pesto alla Genovese 3	Cashew nuts	+
Dairy products		
Milkshake pistachio-cocos	Pistachio pulp	-
Sweets/ Cookies		
Marzipan	Almonds	-
Lokum (Turkish delight)	Pistachios	-
Gingerbread	Hazelnuts, walnuts, almonds, cashew nuts, apricot kernels	+
Spreads		
Bear's garlic spread	Pine nuts	-
Organic mixed nut butter	Peanut, hazelnut, almond, cashew	+

4.4.7 Summary

The developed real-time PCR system targeting part of the cDNA coding for the Ana o3 cashew nut allergen was shown to be a highly specific and sensitive method for the detection of potential allergenic cashew nut ingredients in food. Synthetic oligonucleotides (primers and probe) were designed for the specific detection of a 103 bp long fragment. In addition, a 529 bp amplicon containing the 103 bp target sequence as an internal region was generated by specific primers. This amplicon served as standard DNA for calibration curves and inhibition control in sample analysis.

The specificity of the system was tested successfully with DNA of more than 50 different plant species. No false positive signals were detected either with phylogenetically related organisms or further allergenic tree nuts. In contrast to a previously described real-time PCR cashew detection method [109] even pistachios as a member of the same family (Anacardiaceae) did not result in false positive reactions thus allowing differentiation of allergenic cashew proportions from potentially present amounts of pistachio in food.

Assessment of the sensitivity was based on dilution series of genomic DNA extracted from cashew and dilution series of PCR amplicons (529 bp). Reproducible detection of 0.5 pg genomic DNA and 10 copies of the synthetic amplicon was successfully performed with the new real-time PCR method. In addition, the dynamic range of the linear correlation between signal and amount of analyte over seven orders of magnitue was shown. The limit of detection (LOD) as a measure of the method's applicability to a real food matrix was assessed using spiked pesto genovese. A proportion of 2 mg/kg cashew could be detected. Analysis of commercial food samples demonstrated the applicability of the method to different food matrices. All cashew ingredients could be detected and the absence of cashew in non-declared products could be confirmed.

This assay might be a valuable tool both for the enforcement of labeling requirements concerning potentially allergenic ingredients in food and for tracking and localization of sources of contaminations with cashew nut in industrial production environments.

5 Summary

DNA-based methodologies have become an integral part of food and feed analysis and are commonly applied in many different fields such as detection of allergens, GMO analysis, food authentication or detection of microorganisms (food pathogens).

Recent harmonization of regulatory provisions concerning the use of GMO and GMO-derived material for food and feed purposes gave an impulse to the authorization of various new GM events. Official methods required to control the compliance of these regulations were provided by the European Commission's Reference Laboratory. This results in a bulk of analytical approaches when performed with these single event-specific detection methods. Therefore, the method development performed in this study was focussed on new strategies, combining different demands of GMO analysis such as the steadily growing number of authorized and unauthorized GMO or the need for quantitative assessment of tolerated thresholds.

The new ligation-dependent probe amplification (LPA) technique offers an alternative strategy to standard multiplex PCR approaches for GMO analysis due to its modular character. A ligation of synthetic probes in the presence of target DNA is required for the subsequent competitive amplification using the universal primer binding sites of the generated ligation products. The amplicons have characteristic lengths for each target sequence which enables the simultaneous separation and detection of the amplicons by capillary electrophoresis via laser-induced fluorescence.

The modular system was designed to target GMO at different levels of specificity: the regulatory sequence of the Cauliflower Mosaic Virus (CaMV 35S) promotor, the construct junction between the CaMV 35S promoter and the *pat*-gene, and the event-specific junction regions of maize MON 810 and Roundup Ready soya were included as well as suitable reference genes in the species maize, soya and rapeseed. The specificity and sensitivity of the LPA system was examined with mixtures of DNA extracts from non-transgenic rapeseed, maize and soya, certified reference flour standards from maize MON 810 and Roundup Ready soya with defined proportions of transgenic material and synthetic hybrid amplicons containing the rapeseed-specific target and the LibertyLink™-specific junction region P-35S/*pat* gene. The simultaneous detection of all seven target sequences in one reaction was possible by the LPA approach. The sensitivity for the detection of 0.1 % GMO-derived material was obtained by optimal probe concentration and application of competitor oligonucleotides limiting signal intensity of the reference targets.

In addition, a flexible LPA system for the event-specific quantification of different GM-maize lines was developed by targeting the junction region of transgenic and plant DNA of the recombinant maize events MON 810, Bt 11 and Bt 176 and the *HMGa*-maize reference gene. Mixtures of DNA extracted from certified reference materials with defined GMO proportions between 0.1 % and 5 % transgenic material and negative controls free of GMO were used to prove the specificity and sensitivity of the LPA approach. Again, a maize competitor was applied to allow sensitive determination of 0.1 % transgenic material. To demonstrate the potential for relative quantification by the LPA system calibration curves were generated by plotting the ratio of peak heights from the transgenic target and the reference gene against the defined GMO content of the standard material. Good linear correlations were obtained by simultaneous assessment of the three recombinant maize lines but relative standard deviations did not fulfill method performance criteria accepted for GMO analysis methods. Further experiments should be performed to examine the reproducibility of the ligation reaction.

The development of analytical methods suitable for the specific detection of allergenic components in food products is essential due to the increasing trend of food allergies and the variety/diversity of different food allergens, which have to be labeled to inform and to protect the consumer. Since the entering into force of the labeling directive further food components have been added: lupine and molluscs demonstrate the need to support the compliance of regulatory provisions by extremely sensitive methods allowing multitarget detection. DNA-based methods offer an alternative device to the established protein based ELISA assays due to the high specificity and sensitivity of the approach. LPA technique was applied to allergen analysis, but also established real-time PCR technology was used to develop suitable detection methods.
The developed LPA system for the identification of allergenic components in food allowed the simultaneous detection of DNA from peanut, cashew, pecan nut, pistachio, hazelnut, sesame seeds, macadamia nut, almond, walnut and brazil nut in a single reaction. The designed synthetic hybridization probes were successfully tested for specificity with DNA from more than 50 different species. However, almond probes showed cross reactivity to a few phylogenetically related species, which can be overcome by further characterization of the almond target sequence and a new design of the probes. The sensitivity of the method was assessed by determination of practical limits of detection in different food matrices. The LOD, defined as the lowest concentration of analyte that could be reliable detected, was determined to 5 mg/kg for single allergens. These results were comparable to real-time PCR methods and ELISA approaches. The application to different food matrices was shown by analysis of commercially available food samples.

A novel real-time PCR system for the specific and sensitive detection of DNA from cashew nut was developed targeting a 103 bp part of the cDNA coding for the Ana o3 cashew nut allergen. No cross reactivity was observed by testing phylogenetically related organisms or further allergenic tree nuts and no false positive signals were detected by analysis of DNA from 50 different species, thus confirming the specificity of the method. Suitable sensitivity for the detection of traces of allergenic components in food was shown by reproducible detection of 0.5 pg genomic DNA and 10 copies of synthetic target sequence. In addition, the practical limit of detection by analysis of a real food matrix was determined to 2 mg/kg cashew nut in pesto genovese. The applicability of the method to different food matrices was demonstrated by successful determination of cashew nut ingredients or confirmation of the absence of cashew nut in retail samples.

6 Zusammenfassung

Molekularbiologische Nachweisverfahren sind ein fester Bestandteil in vielen Bereichen der modernen Lebensmittelanalytik geworden. Der Nachweis von Lebensmittelallergenen, die GVO-Analytik, Authentizitätsbestimmungen durch Tier- und Pflanzenartendifferenzierung und der Nachweis lebensmittelpathogener Mikroorganismen werden mittels DNA-basierter Methoden routinemäßig durchgeführt.

Die jüngste Harmonisierung der Gesetzgebung zur Rückverfolgbarkeit, Zulassung und Kennzeichnung von gentechnisch veränderten Organismen führte zu einer Reihe von Neuzulassungen einer Vielzahl neuer GVO-Events. Das gemeinschaftliche Referenzlaboratorium der EU stellt die erforderlichen offiziellen Nachweismethoden der Öffentlichkeit zur Verfügung. Diese einzelnen eventspezifischen Methoden führen zu einer umfangreichen und aufwändigen GVO-Analytik. Daher wurden in dieser Arbeit bei der Methodenentwicklung neue Strategien verfolgt, welche die verschiedenen Anforderungen bei der Analytik von GVO, wie die steigende Anzahl an zugelassenen und nicht zugelassenen GVO oder die quantitative Bestimmung der tolerierten Schwellenwerte, berücksichtigen.
Die neuartige Technik der ligationsabhängigen Amplifizierung von Hybridisierungssonden (LPA) bietet aufgrund ihres modularen Aufbaus eine Alternative zu Multiplex PCR-Methoden. Durch Ligation von synthetischen DNA-Sonden in Gegenwart der Ziel-DNA entstehen Ligationsprodukte, welche im Anschluss in einer kompetitiven PCR mit nur einem Primer-Paar vervielfältigt werden. Die Amplikons weisen eine für die Target-DNA charakteristische Länge auf und können so gleichzeitig über eine Kapillargelelektrophorese aufgetrennt und mittels laserinduzierter Fluoreszenz detektiert werden.
Für den Nachweis von GVO auf unterschiedlichen Spezifitätsstufen wurde ein modulares System entwickelt. Dafür wurden Sonden entworfen, die den Nachweis der regulatorischen Sequenz des Blumenkohlmosaikviruspromotor (CaMV 35S), den konstruktspezifischen Übergang vom CaMV 35S Promotor zum *pat*-Gen und den eventspezifischen Nachweis von Mais MON 810 und Roundup Ready Soja ermöglichen. Daneben konnten geeignete Referenzgene der Spezies Mais, Soja und Raps in das System integriert werden. Die Spezifität und Sensitivität des LPA-Systems wurde mit DNA-Mischungen aus nicht transgenem Raps, Mais und Soja, zertifizierten Referenzmaterialien mit definiertem GVO-Gehalt an Mais MON 810 und Roundup Ready Soja und synthetischen Hybridamplikons, die je eine Kopie der rapsspezifischen Sequenz und des LibertyLink™-spezifischen Übergangs P-35S/*pat*-Gen enthalten, untersucht. Die

Methode erlaubt den gleichzeitigen Nachweis aller sieben Zielsequenzen in einem Reaktionsansatz. Durch Optimierung der Sondenkonzentrationen und Verwendung von Kompetitor-Oligonukleotiden zur Limitierung der Signalintensitäten der Referenzgene konnte eine Nachweisempfindlichkeit von 0,1% gentechnisch verändertem Material erreicht werden.

Daneben wurde ein flexibles LPA-System zum eventspezifischen Nachweis und zur Quantifizierung der GV-Maislinien MON 810, Bt 11, Bt 176 sowie des maisspezifischen Referenzgens *HMGa* entwickelt. DNA-Mischungen aus zertifizierten Referenzmaterialien mit GVO-Anteilen von 0,1% bis 5% und nicht transgenem Material wurden untersucht, um die Spezifität und Sensitivität zu zeigen. Durch die erneute Verwendung eines DNA-Kompetitors für das Referenzgen konnte eine Empfindlichkeit von 0,1% transgenem Anteil in der Probe erreicht werden. Zur Überprüfung der Eignung der Methode zur relativen Quantifizierung von GVO wurden Kalibriergeraden erstellt. Das Verhältnis der Peakhöhen der transgenen Targets zu der des Referenzgens wurde gegen den zertifizierten GVO-Gehalt der Standards aufgetragen. Die aus der gleichzeitigen Analyse der drei rekombinanten Maislinien resultierenden Geraden zeigten eine gute lineare Korrelation, allerdings erfüllen die relativen Standardabweichungen nicht die Kriterien des ENGL bzgl. GVO-Methoden. Weitere Untersuchungen sind notwendig, um die Reproduzierbarkeit speziell der Ligationsreaktion zu analysieren.

Die steigende Häufigkeit an Lebensmittelallergien und die möglichen gesundheitsgefährdenden Folgen eines unbemerkten Verzehrs allergener Zutaten haben zur Einführung verbesserter Kennzeichnungsvorschriften durch die EU-Richtlinie 2003/89 geführt. Die Erweiterung der kennzeichnungspflichtigen Zutaten auf Lupine und Mollusken zeigt, dass auch im Bereich der Allergenkennzeichnung spezifische und sensitive Methoden zum Nachweis von allergenen Lebensmittelzutaten erforderlich sind, um die Kontrolle der gesetzlichen Kennzeichnungsvorschriften zu gewährleisten. Hier bieten spezifische und sensitive molekularbiologische Methoden eine Alternative zu den etablierten immunochemischen ELISA-Verfahren. Die neuartige LPA-Technik wurde ebenso wie die etablierte Real-time PCR Technologie angewandt, um geeignete Nachweisverfahren zu entwickeln.

Das entwickelte LPA-System zur Identifizierung von allergenen Bestandteilen in Lebensmitteln erlaubt die gleichzeitige Detektion von DNA aus Erdnuss, Cashewnuss, Pecannuss, Pistazie, Haselnuss, Sesam, Macadamianuss, Mandel, Walnuss und Paranuss in einem Reaktionsansatz. Die Spezifität der synthetischen Hybridisierungssonden wurde gegenüber mehr als 50 verschiedenen Spezies bestätigt. Lediglich die Mandelsonden zeigten eine Kreuzreaktion gegenüber einigen

phylogenetisch nah verwandten Arten, welche jedoch durch nähere Charakterisierung der Mandel-Zielsequenz und Neudesign der Sonden eliminiert werden kann. Verschiedene dotierte Lebensmittelmatrices wurden zur Bestimmung der Empfindlichkeit der Methode untersucht. Die Nachweisgrenze, d.h. die kleinste Konzentration, die reproduzierbar positiv detektiert werden konnte, wurde mit 5 mg/kg für einzelne Allergene bestimmt. Diese Ergebnisse sind mit Real-time PCR Methoden und ELISA-Verfahren vergleichbar. Durch Untersuchung kommerziell erhältlicher Lebensmittel konnte die Anwendbarkeit der Methode auf verschiedene Lebensmittelmatrices gezeigt werden.

Eine neu entwickelte Real-time PCR Methode zum spezifischen und sensitiven Nachweis von Cashew-DNA detektiert einen 103 bp langen Abschnitt der cDNA, die für das Cashew-Allergen Ana o3 kodiert. Es konnte keine Kreuzreaktion bei der Untersuchung phylogenetisch verwandter Organismen oder weiterer allergener Baumnüsse festgestellt werden. Die Analyse von DNA aus 50 weiteren Spezies ergab keine falsch positiven Signale, was die Spezifität der Methode bestätigt. Die absolute Nachweisgrenze konnte mit 0,5 pg genomische DNA bzw. 10 Kopien der synthetischen Zielsequenz bestimmt werden. Die praktische Nachweisgrenze wurde mit 2 mg/kg Cashewnuss durch Untersuchung einer gespikten Matrix Pesto Genovese bestimmt. Die Anwendbarkeit der Methode auf verschiedene Lebensmittelmatrices konnte durch erfolgreichen Nachweis von Cashew-Anteilen bzw. durch Bestätigung der Abwesenheit von Cashewnuss in kommerziellen Handelsproben gezeigt werden.

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