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DNA-based quantification of food allergens:

Comparative assessment of quantification methods and application of the standard addition

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Content

1 Summary

Food allergies constitute worldwide a health concern for approximately 3 to 6% of consumers. Accordingly, frameworks concerning the labeling of allergenic foods are being implemented by regulatory authorities. To be able to distinguish unintentional contamination and intentional addition of these components and to monitor current as well as possible future thresholds for allergens in food, quantitative methods are required.

The objective of this thesis was the comparative assessment of the performance of different DNA-based quantification strategies. As validation protocols and harmonized standard materials for trace analysis of allergenic foods are lacking, uniform experimental conditions were set up. The validation was performed with the pre-defined performance parameters recovery, repeatability, reproducibility, and sensitivity. On this basis, a comparative assessment of DNA-based methods determining sesame as analyte using (i) a matrix-adapted calibration, (ii) an internal standard material and (iii) a standard addition was performed. As reference material boiled sausage spiked with sesame was used. A quantification of sesame in self-prepared muffin dough and muffins was performed to demonstrate the applicability of the methods to the routine analysis of foods.

As the standard addition emerged as the most appropriate approach, a tetraplex real-time PCR was combined with this quantification method. Thus, the method's disadvantage of being very laborious could be diminished by the simultaneous determination of the allergenic foods celery, soy bean, brown mustard, and white mustard. The functionality of the established tetraplex real-time PCR was proven by analysis of tenfold DNA dilution series. The determined PCR efficiency and coefficient of variation reflect the linearity for each of the examined parameter. The validation was performed using boiled sausages, and the performance criteria recovery, repeatability, robustness and sensitivity were determined. The sensitivity, expressed as the limit of quantification, was for all parameters in the range of 2 to 40 mg/kg, thus underlining the applicability of the method for trace analysis of allergenic foods. The suitability for the allergen determination in commercially available food samples was proven by screening of four different foods either containing one or more of the allergenic foods or at least being labeled with a "may contain …" hint of the allergenic foods.

Subsequent to the screening, the quantification of the allergens was performed, indicating the method's applicability in routine analysis for both, screening and quantification.

The applicability of the standard addition method, proved in this thesis to generate true and precise results, was confirmed by a ring trial organized by the working group for food allergens according to § 64 of the German Food and Feed Act and the subsequent implementation of this quantification method in the collection of § 64 methods of analysis. The practicability of the established tetraplex real-time PCR was also demonstrated by the implementation of the approach in combination with a matrix-matched calibrator to this official collection of analytical methods.

Zusammenfassung

Lebensmittelallergien stellen weltweit für ungefähr 3 bis 6% der Verbraucher ein gesundheitliches Problem dar. Deshalb werden rechtliche Rahmenbedingungen zur Kennzeichnung potenziell allergieauslösender Lebensmittel implementiert. Um einen unbeabsichtigten Allergeneintrag von einer absichtlichen Zugabe unterscheiden zu können oder die Überwachung von aktuellen oder möglichen künftigen Schwellenwerten zu ermöglichen, sind quantitative Analysenmethoden erforderlich.

Zielsetzung der vorliegenden Arbeit war, die Leistungsfähigkeit verschiedener DNA-basierter Quantifizierungsstrategien zu vergleichen. Aufgrund fehlender Validierungs-vorschriften und harmonisierter Standardmaterialien für die Spurenanalytik im Bereich der Lebensmittelallergene wurden einheitliche experimentelle Rahmenbedingungen geschaffen. Auf Basis dieser Rahmenbedingungen wurde eine Validierung mit vordefinierten Leistungskriterien durchgeführt. Diese Kriterien waren die Wiederfindung, die Wiederholbarkeit, die Reproduzierbarkeit und die Sensitivität. Mit Sesam als Analyt wurden verschiedene DNA-basierte Methoden verglichen. Die Quantifizierung wurde (i) mittels matrix-adaptierter Kalibrierung, (ii) unter Verwendung eines internen Standards und (iii) mit Hilfe einer Standardaddition durchgeführt. Für die Ermittlung der Leistungskriterien wurde die Validierung mit dotiertem Brühwurstmaterial durchgeführt. Die Anwendbarkeit der Quantifizierungsmethoden für die Untersuchung von Routineproben wurde mit der Untersuchung von selbsthergestelltem Muffinteig und daraus gebackenen Muffins gezeigt.

Die Standardaddition erwies sich, auf Basis der ermittelten Validierungsdaten, als die geeignetste, wenngleich auch als die aufwendigste Quantifizierungsmethode, weshalb diese mit einer tetraplex real-time PCR kombiniert wurde. Der Vorteil der tetraplex PCR liegt darin, die vier allergenen Lebensmittel Soja, Sellerie, brauner Senf und weißer Senf gleichzeitig bestimmen zu können und so den Nachteil der aufwendigen Methode kompensieren zu können. Die Funktionalität der tetraplex real-time PCR wurde durch die Analyse einer dekadischen Verdünnungsreihe der Analyten untersucht. Die daraus ermittelte PCR Effizienz und das Bestimmtheitsmaß geben die Linearität für jeden der analysierten Parameter wieder. Die Leistungsparameter Wiederfindung, Wiederholbarkeit, Robustheit und Sensitivität der tetraplex real-time PCR wurden in dotierten Brühwürsten validiert. Die Sensitivität, angegeben als Bestimmungsgrenze,

lag für alle untersuchten Parameter im Bereich von 2 bis 40 mg/kg, was die Tauglichkeit der Methode zur Spurenanalytik allergener Lebensmittel unterstreicht. Die Anwendbarkeit der Analysenmethode für die Routineanalytik konnte durch Untersuchung vier kommerziell erhältlicher Proben gezeigt werden, die eines oder mehrere der mittels der tetraplex real-time PCR erfassten allergenen Lebensmittel als Zutat enthielten, oder zumindest in Form einer Spurenkennzeichnung deklariert waren. Die anschließende Quantifizierung von Proben, die sich im Screening als positiv erwiesen hatten, zeigte die Eignung der Methode in der Routineanalytik, sowohl für den Nachweis als auch die Bestimmung der allergenen Lebensmittel.

Die Eignung der Standardaddition, richtige und präzise Ergebnisse zu liefern, wurde durch die Ergebnisse eines Ringversuchs der § 64 Arbeitsgruppe „Lebensmittelallergene“ und die anschließende Aufnahme in die Methodensammlung nach § 64 des Lebensmittel- und Futtermittelgesetzbuches bekräftigt. Die Anwendbarkeit der etablierten tetraplex real-time PCR wurde ebenfalls durch die Aufnahme in die Methodensammlung nach § 64 des Lebensmittel- und Futtermittelgesetzbuches unterstrichen.

2 Introduction and objectives

Food allergy is a significant public health concern throughout the world as it affects about 3 to 6% of the population (Sicherer, 2011). Symptoms elicited by a food allergy may range from mild to severe or even be life-threatening (Boye et al., 2012). Although several strategies with the aim of long-term treatment and possible cures for established food allergies are being discussed, the current standard of care is the strict avoidance of allergenic foods (Hoffmann-Sommergruber, 2016; Wang and Sampson, 2012). In order to provide affected consumers with the means to avoid foods that might lead to adverse immunologic responses, governments all over the world have implemented regulatory frameworks concerning the labeling of allergenic ingredients (Gendel, 2013). In the European Union (EU), the most common allergenic ingredients need to be labeled according to Directive No. 1169/2011 on the provision of food information to consumers in order to protect the consumers from unintentional consumption (EU, 2011). In Annex II of this directive, 14 foods and food groups are listed for which labeling is mandatory if they are used for the production or preparation of foods and are present in the product. However, this regulatory provision does not apply to traces of allergenic foods, which entered the product unintentionally through cross-contact. Regulation 2002/178/EC states that food shall not be placed on the market if it is unsafe (EU, 2002). To ensure their duty of care, food manufacturers often use “may contain” statements. The excessive usage of such precautionary labeling leads to uncertainty amongst consumers (Kerbach et al., 2009). Consequently, thresholds concerning allergens arising from cross-contacts are being discussed (Richter et al., 2012), as the implementation of such mandatory thresholds might help to reduce the uncertainty of consumers. To monitor the compliance with these potential future thresholds, both food industry and food safety authorities are in need of methods for the quantitative analysis of food allergens.

For the analysis of food allergens, protein- and DNA-based methods are available. Immunochemical methods, mainly enzyme linked immunosorbent assays (ELISAs), are widely used to quantify food allergens, as the methods do not require expensive equipment, and various kits are commercially available (Kerbach et al., 2009; Schubert-Ullrich et al., 2009). Recently, mass spectrometry-based methods for single and multiple detection and quantification of various allergens have been developed

(Monaci et al., 2018). DNA-based polymerase chain reaction (PCR) is used mainly as screening tool to detect the presence of allergenic components, whereas real-time PCR methods offer the possibility to generate quantitative data based on the initial copy numbers. Several real-time PCR methods have already been published for the detection of sesame (Brzezinski, 2007; Mustorp et al., 2008; Schöringhumer et al., 2009), celery (Fuchs et al., 2012; Hupfer et al., 2007; Mustorp et al., 2008), soy bean, (Gryson et al., 2008; Köppel et al., 2010) white mustard (Fuchs et al., 2010) and brown mustard (Palle-Reisch et al., 2013).

Kits allowing for a matrix-adapted quantification of sesame (R-Biopharm, 2018d), soy bean (R-Biopharm, 2019a), celery (R-Biopharm, 2018b) and the detection of mustard (R-Biopharm, 2018c) are commercially available. Meanwhile, a multiplex kit for the simultaneous detection and matrix-adapted quantification of soy bean, celery and mustard has become available (R-Biopharm, 2018a). The quantification is achieved by co-analyzing of an external calibrator material (R-Biopharm, 2019b).

For the detection and quantification of soy bean and white mustard, respectively, singleplex real-time PCR systems based on an external calibration have been published by the Federal Office of Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL). The disadvantage arising from the fact that this approach is matrix-dependent has been compensated for by using identical sample and calibration matrices (BVL, 2013). In routine analysis, however, this might be difficult to achieve, owing to the high diversity of potential food matrices.

Köppel et al. (2010) established a tetraplex real-time PCR system to simultaneously detect and quantify DNA from celery and soy bean in addition to hazelnut and peanut. This tetraplex real-time PCR system was extended to a hexaplex system for the detection and quantification of DNA from soy bean, celery, white mustard, cashew, peanut and hazelnut (Köppel et al., 2012). However, the method does not allow for the quantification of the allergens in food, because the content of the allergenic food cannot be extrapolated from the ratio of the respective species-specific DNA to the total DNA content (Köppel et al., 2012).

Besides the quantification using an external calibration as applied in the commercially available kits, two other DNA-based quantification approaches have been developed with the aim to calculate quantitative data from the cycle threshold values while overcoming the dependency on appropriate standard materials that correspond to the

respective food matrices. One of the quantification methods is based on the addition of a unique internal standard material (Hirao et al., 2006), the other on the principle of standard addition (Eugster, 2010). Meanwhile, a third quantification approach has been published based on addition of a threshold-calibrated competitor sequence that is co-analyzed by competitive real-time PCR (Holzhauser et al., 2014; Ladenburger et al., 2018).

Due to the lack of reference materials and standardized validation protocols for DNA-based quantification methods for food allergens, a comparison of the performances of the different quantification methods has not been possible so far. Therefore, the objective of the studies underlying this thesis was to set up experimental framework conditions that allow a comparative assessment of the different quantification approaches. Materials tested for homogeneity were analyzed using three different quantification methods, and the results were evaluated on the basis of uniform assessment criteria. The suitability of the methods to quantify sesame in model foods was investigated through the analysis of muffin dough spiked with different amounts of sesame and of the respective baked muffins. Considering the performance criteria, the standard addition method evolved as the most promising method, although it is very laborious. To overcome this disadvantage, a tetraplex real-time PCR method was established allowing the simultaneous quantification of trace amounts of celery, soy bean, white mustard and brown mustard. The quantification method was based on a standard addition procedure in order to be independent from an external calibration. The method development was performed using boiled sausages as matrix; the suitability of the method was demonstrated by the investigation of commercially available foods containing celery, mustard and/or soy bean either as ingredient or mentioned in a precautionary “may contain…” labeling.

3 Background and methods

3.1 Food allergy

3.1.1 Definition

Food sensitivities represent any abnormal clinical response associated with the ingestion of food (Figure 1). Food sensitivities are divided into toxicological reactions, such as to bacteria, and non-toxicological reactions, the so-called hypersensitivities. Hypersensitivity encompasses both food intolerance, caused by a non-immunological response, and food allergy, which is immunologically mediated.

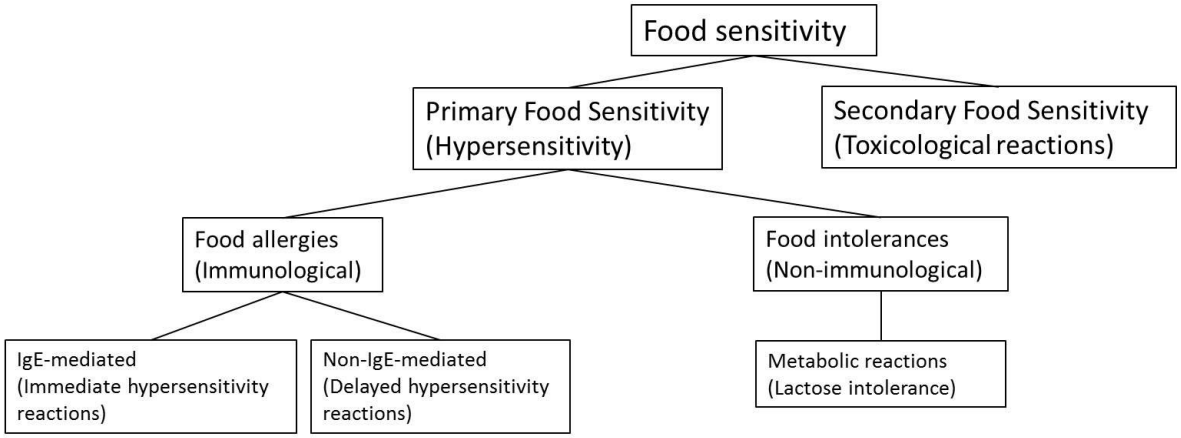

Figure 1: Classification of food sensitivities (modified from Johansson et al. (2003))

Food intolerances can result from metabolic disorders such as for example phenylketonuria or lactose intolerance (Boye et al., 2012). A deficiency of the enzyme lactase that hydrolyses lactose into glucose and galactose, which are then resorbed in the small intestine, leads to a situation where the lactose remains in the gut and causes symptoms like abdominal cramping (Wüthrich, 2008).

Most food allergies are immunoglobulin E (IgE)-mediated (Ebo and Stevens, 2001). IgE-mediated allergies result in immediate hypersensitivity reactions, with the symptoms occurring within a few minutes to a few hours (Taylor, 2001). More than 170 foods have been reported to be involved in immediate hypersensitivity reactions (Taylor and Lehrer, 1996). Non IgE-mediated food allergies trigger delayed hypersensitivity reactions, whereby the symptoms occur after several hours or even days. The primary disorders are food protein-induced enterocolitis, food protein-induced proctitis/proctocolitis and enteropathy (EFSA, 2004). An example is the gluten-

sensitive enteropathy, also known as gluten intolerance or celiac disease, an abnormal immunological response to gluten/gliadin (Boye et al., 2012).

3.1.2 Mechanism

IgE-mediated food allergies develop in two stages, the sensitization stage (Figure 2A) and the allergic reaction (Figure 2B). In the sensitization phase, the antigen is presented to the immune system for the first time and leads to the stimulation of the production of IgE antibodies in B-cells. The IgE antibodies then bind to the surface of mast cells in various tissues and to basophils in the blood (Taylor, 2001). These mast cells and basophils are packed with granules that contain inflammatory mediators, like histamine, cytokines and leukotrienes (Boye et al., 2012). The second phase is the re-exposure where the previously sensitized person is again exposed to the allergen. The allergens cross-link two IgE antibodies that are anchored on the surface of the mast cells and basophils, and trigger the release of the mediators leading to the allergic response (Taylor, 2001).

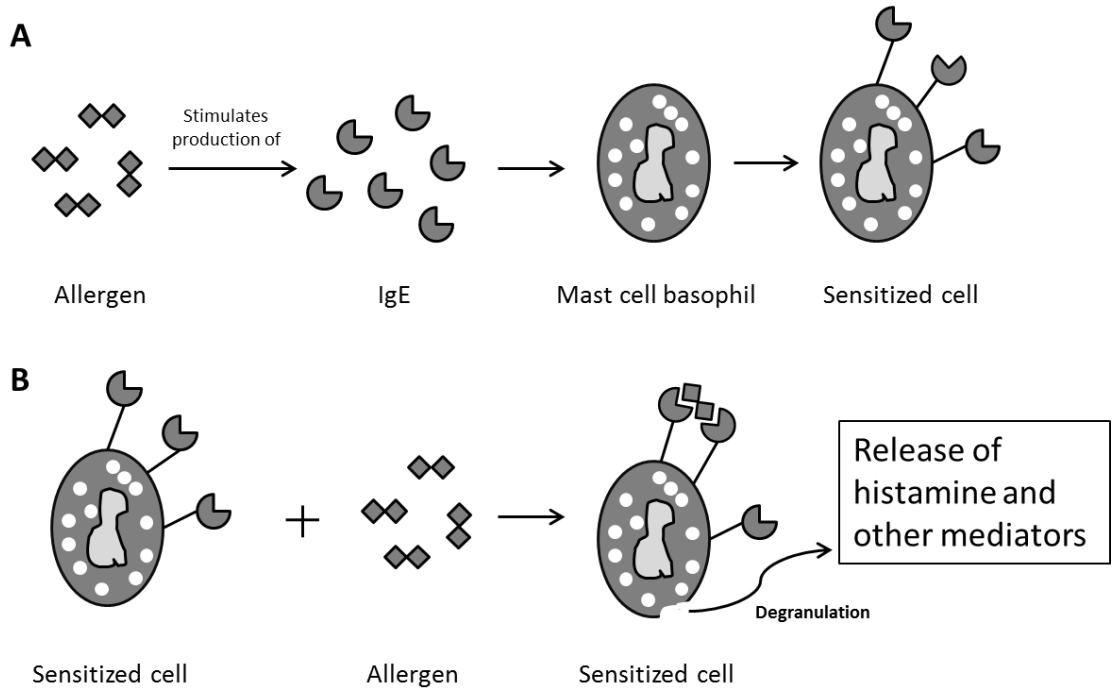

Figure 2: Mechanism of IgE-mediated allergic reactions: (A) After the first exposure to the allergen the individual is sensitized. The sensitization stimulates the production of allergen-specific IgE-antibodies that bind to receptors of the mast cell basophils resulting in sensitized cells. (B) After a second exposure to the allergen, the allergens cross-link two linked antibodies of the sensitized cells which leads to the degranulation and the release of histamine and other chemical mediators of the allergenic response (adapted from Taylor (2001)).

3.1.3 Symptoms

The intensity of the symptoms of allergic reactions can vary from mild to severe and can even be life-threatening. The severity of the symptoms depends on the degree of sensitization, the amount of consumed allergenic food (Taylor, 2001) and various other factors, e.g. the degree of food processing (Maleki, 2004), the environment, and physiological conditions (Sicherer and Sampson, 2014). The manifestation of the symptoms may appear as gastrointestinal (nausea, vomiting, diarrhea, abdominal pain or colic), cutaneous (hives, eczema or atopic dermatitis, angioedema or pruritus) or respiratory disease (rhinitis or asthma), severe hypotension, or even anaphylactic shock (Taylor, 2001).

3.1.4 Food allergens

Although foods contain various different proteins, more than 65% of all plant allergens belong to four main families: the prolamin superfamily, the cupin superfamiliy, the Bet v 1 family and the profilins (Hoffmann-Sommergruber and Mills, 2009). Proteins of the prolamin superfamiliy are mainly seed storage proteins (gliadins and glutenins), non-specific lipid transfer proteins, α-amylase/protease inhibitors and 2S albumins. Allergens from the prolamin superfamily occur in various foods, e.g. cereals, nuts, peanuts, yellow mustard, peach or apple (Hoffmann-Sommergruber and Mills, 2009). Members of the cupin superfamily are the seed storage proteins 7S/8S and the 11S globulins. The 7S/8S globulins include allergens from peanut, walnut, sesame and various others, the 11S globulins from peanut, soy, Brazil nut and buckwheat (Mills et al., 2004). Profilins are regulatory proteins and occur in e.g. apple, hazelnut, peanut, celery or wheat. The first identified Bet v 1-related protein was the major inhalant allergen birch pollen. Bet v 1-related proteins are mainly plant defense proteins and are present in a variety of plants. In this case, the primary sensitization is usually triggered through contact with the inhalant allergen birch pollen and re-exposure, and the subsequent allergenic reactions are often caused by the consumption of various foods (fruits, nuts or vegetables) containing Bet v 1-related proteins (Hoffmann-Sommergruber and Mills, 2009).

The most prevalent protein families of animal food allergens are tropomyosins (crustaceans and mollusks), parvalbumins (fish) and caseins (mammalian milk) (Jenkins et al., 2007). Besides, there are a number of other food allergens from animal

origin like ß-lactoglobulin and α-lactalbumin from whey and ovotransferrin, ovomucoid, ovalbumin, lysozyme and α-livitin from hen's egg (Jędrychowski, 2008). The allergenicity of a complex food is generally not caused by a single protein, but by the combination of potentially allergenic proteins present in the food (EFSA, 2004).

3.1.5 Effect of food processing on the allergenicity

Food is processed in order to improve the digestibility and to ensure the hygienic integrity at home, in restaurants or by the food industry (EFSA, 2004). The processing of food can affect the structural and chemical properties of the contained proteins, and thereby alter the food's allergenic potential (Paschke, 2009).

Common processing techniques applied in the food industry that have been associated with an influence on the allergenicity include thermal treatment, acid or enzymatic hydrolysis, fermentation, physical processing (such as high pressure treatment or extrusion), chemical treatments (such as changes in the pH), changes in the composition (addition of preservatives) or combinations of these (EFSA, 2004). The influence of the different processing techniques on the structural properties of a food allergen and, in consequence, on the allergenicity is difficult to predict, considering the complexity of interactions between different contained allergens, and additionally between the allergens and the many diverse food matrices (Mills et al., 2009). The allergenicity of a complex food may be decreased, remain unchanged, or even be increased by food processing (EFSA, 2004).

Verhoeckx et al. (2015) reviewed the impact of processing on the allergenic potential of proteins from various allergenic foods (peanut, tree nuts, cows' milk, hens' eggs, soy, wheat, and mustard) and came to the overall conclusion that processing may influence, but does not abolish the allergenic potential of proteins.

The influence of thermal treatment on the allergenic properties of peanuts has been investigated (Beyer et al., 2001; Koppelman et al., 1999; Maleki et al., 2000). An increase of the allergenicity of roasted peanuts was observed compared to boiled or fried peanuts (Beyer et al., 2001) and compared to raw peanuts (Maleki et al., 2000), whereas no change of allergenicity was previously reported (Koppelman et al., 1999). A decrease of the allergenic potential of roasted hazelnuts compared to raw hazelnuts was observed by Hansen et al. (2003).

3.1.6 Management of food allergy

As there are currently no cures for food allergy, the mainstay of allergen management is the dietary avoidance of foods containing allergens or allergen-derived ingredients in combination with nutritional education (Boye et al., 2012; EFSA, 2004). Patients, their caregivers, or both need to be educated about food allergen avoidance (i.e. reading food labels, avoiding high-risk situations e.g. buffets, early recognition of allergic symptoms, and early management of an anaphylactic reaction) (Sampson, 1999).

3.2 Legislation

As allergic patients are dependent on the avoidance of the consumption of the allergenic substances, they need accurate information on the presence of allergens in foods. Governments all over the world implemented various regulations regarding the declaration of allergens. Eggs, milk, peanuts and cereals containing gluten have to be labeled in Japan, South Korea, USA, Canada, EU, Australia and New Zealand. Whereas in Japan and South Korea, buckwheat is part of the list, in the USA, tree nuts, crustaceans, fish and soy bean must additionally be labeled. In Canada, Australia and New Zealand, the US list is extended by sesame seed and sulfite. In Europe, Regulation No. 1169/2011 on the provision of food information determines that accurate information about the composition of prepacked and non-prepacked foods must be provided to the consumer. The Canadian and Australian list is extended by celery, mustard, mollusks and lupine to a total of 14 ingredients that most frequently lead to allergic reactions. The allergens requiring labeling in the EU are listed in Regulation No. 1169/2011 (EU, 2011; Annex II) and are depicted in Table 1.

According to the Regulation No. 1169/2011, only allergens intentionally added to a food have to be declared. However, hidden allergens entered by cross-contacts are known to be potentially hazardous to sensitized persons (Jäger and Vieths, 2008). Cross-contacts can occur when different foods are produced and stored in the same facility, e.g. resulting from ineffective cleaning (Buchanan, 2008), from dust formation (e.g. flour or milk powder), or the use of common equipment and/or environmental transfer (Kerbach et al., 2009).

As Regulation 2002/178/EC (EU, 2002) states that food shall not be placed on the market if it is unsafe and Regulation No. 1169/2011 (EU, 2011) lacks in addressing allergenic substances unintentionally entered to the product, manufacturers can voluntarily add “may contain” statements to the food labeling, to ensure their duty of care. This excessively practiced precautionary labeling leads to uncertainty amongst consumers and negatively affects their confidence in the safety of foods (Kerbach et al., 2009). As a result, the allergic consumer possibly unnecessarily restricts his choice of foods.

Table 1: Annex II of EU Regulation No. 1169/2011: Substances and products causing allergies or intolerances (EU, 2011)

1. Cereals containing gluten, namely: wheat, rye, barley, oats, spelt, kamut or their hybridized strains, and products thereof, except:
    (a) wheat based glucose syrups including dextrose[a];
    (b) wheat based maltodextrins[a];
    (c) glucose syrups based on barley;
    (d) cereals used for making alcoholic distillates including ethyl alcohol of agricultural origin;
2. Crustaceans and products thereof;
3. Eggs and products thereof;
4. Fish and products thereof, except:
    (a) fish gelatine used as carrier for vitamin or carotenoid preparations;
    (b) fish gelatine or Isinglass used as fining agent in beer and wine;
5. Peanuts and products thereof;
6. Soybeans and products thereof, except:
    (a) fully refined soybean oil and fat[a];
    (b) natural mixed tocopherols (E306), natural D-alpha tocopherol, natural D-alpha tocopherol acetate, and natural D-alpha tocopherol succinate from soybean sources;
    (c) vegetable oils derived phytosterols and phytosterol esters from soybean sources;
    (d) plant stanol ester produced from vegetable oil sterols from soybean sources;
7. Milk and products thereof (including lactose), except:
    (a) whey used for making alcoholic distillates including ethyl alcohol of agricultural origin;
    (b) lactitol;
8. Nuts, namely: almonds (*Amygdalus communis* L.), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan

nuts (*Carya illinoinensis* (Wangenh.) K. Koch), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*), macadamia or Queensland nuts (*Macadamia ternifolia*), and products thereof, except for nuts used for making alcoholic distillates including ethyl alcohol of agricultural origin;

9. Celery and products thereof;
10. Mustard and products thereof;
11. Sesame seeds and products thereof;
12. Sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/litre in terms of the total SO_2 which are to be calculated for products as proposed ready for consumption or as reconstituted according to the instructions of the manufacturers;
13. Lupine and products thereof;
14. Mollusks and products thereof.

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[a] and the products thereof, in so far as the process that they have undergone is not likely to increase the level of allergenicity assessed by the Authority for the relevant product from which they originated.

Thresholds assessing the risk from allergenic foods at population level can help the food industry and the regulatory authorities to design appropriate food safety objectives to guide risk management (Crevel et al., 2008). In 2008, German experts demanded thresholds for labeling and standards for handling allergenic foods (BfR, 2008). Subsequently, the Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung, BfR) published an opinion on improved allergen labeling of foods for consumers with the conclusion that thresholds cannot be defined yet, due to poor scientific data on doses that might trigger allergic responses. If provisional thresholds were to be determined on the basis of the current state of scientific knowledge, thresholds should range from 0.001 to 0.01% allergenic substances per kg food, depending on the specific allergen (BfR, 2010).

Regulation No. 1169/2011 (article 36) (EU, 2011) wants the Commission to adopt implementing acts on the information on possible and unintentional presence in food of substances or products causing allergies or intolerances. This voluntary information shall not be ambiguous or confusing for the consumer. Although this is clearly stated in the regulation, the Commission has not yet implemented this requirement. Reviewing the European regulations for labeling requirements for food allergens, Popping and Diaz-Amigo (2017) came to the conclusion, that it is not likely to see any threshold levels before 2024, even though the EFSA has called for proposals entitled “Detection and quantification of allergens in foods and minimum eliciting doses in food allergic individuals” with a deadline of 25 October 2017[a].

Considering the current situation for European food information legislation, a pragmatic approach was chosen by the official food control laboratories in Germany in 2014 establishing internal action values. These action values take current analytical experiences and allergologic reference doses into account (Waiblinger and Schulze, 2018). The reference doses have been established by the Australian Allergen Bureau[b] as Voluntary Incidental Trace Allergen Labelling (VITAL) concept (Taylor et al., 2014) that provides reference doses (protein level in mg) based on the evaluation of clinical

[a] https://www.efsa.europa.eu/en/art36grants/article36/170502

[b] http://allergenbureau.net/vital/

data. The main reasons for the German action values are a uniform basis for the appraisal of analytical findings within the different German federal states, definition of uniform target levels (in mg/kg) for the respective allergenic substances for analytical validation and the definition of thresholds to address technically unavoidable contaminations. The action levels for mustard are >5 mg/kg, for celery >20 mg/kg, for soybeans >20 mg/kg and for sesame >10 mg/kg inferred from the reference doses and an assumed daily intake of 100 mg food (Waiblinger and Schulze, 2018).

In Switzerland, an even more pragmatic approach has already been established concerning threshold levels for allergens in foods. Allergenic foods have to be labeled if the amount is above 1000 mg per kilogram or liter food regardless of the source of the allergenic food (EDI, 2016).

The need for thresholds in allergen legislation is underlined by the fact that stakeholders, e.g. the official food control laboratories in Germany or the Australian Allergen Bureau, established action values to give themselves direction in handling allergens in foods. Besides reliable scientific data on relevant allergen doses, another important prerequisite for the establishment of thresholds are reliable and practical analytic tools to determine the relevant allergenic foods in the products.

3.3 Methods for food allergen analysis

In order to ensure the compliance with food labeling legislation, reliable methods for the detection and quantification of food allergens, respectively allergenic foods, are necessary (EFSA, 2004). Different factors need to be considered when choosing an appropriate method: food matrix interference, nature and quantity of the target allergen, the desired level of detection, specificity, and resources and time required for running the assay (Boye et al., 2012). Different approaches have been designed but considering the various requirements, no single method fits all purposes (EFSA, 2004). However, each assay has to permit the unambiguous identification of the allergens (Kirsch, 2009) with a sufficient sensitivity of 1-100 milligram analyte per kilogram food (Poms et al., 2004).

In food allergen analysis, predominantly protein-based methods (mainly enzyme-linked immunosorbent assays (ELISAs) and mass spectrometry (MS) and DNA-based methods (mainly qualitative polymerase chain reactions, PCRs, and real-time PCRs) are applied (Fuchs et al., 2010).

3.3.1 Protein-based methods

ELISAs are the most commonly used method in laboratories for the detection of trace amounts of allergens (Kirsch, 2009). The principle is based on the binding of the allergens or species-specific marker proteins to corresponding antibodies. An enzyme is linked to the antibody in this complex. It converts a chromogenic substrate to a colored product. The absorbance of the product is measured and the concentration is determined by means of a standard curve. Mainly, two different types of ELISAs can be distinguished: the competitive and the sandwich ELISA. The principle of the competitive ELISA is shown in Figure 3.

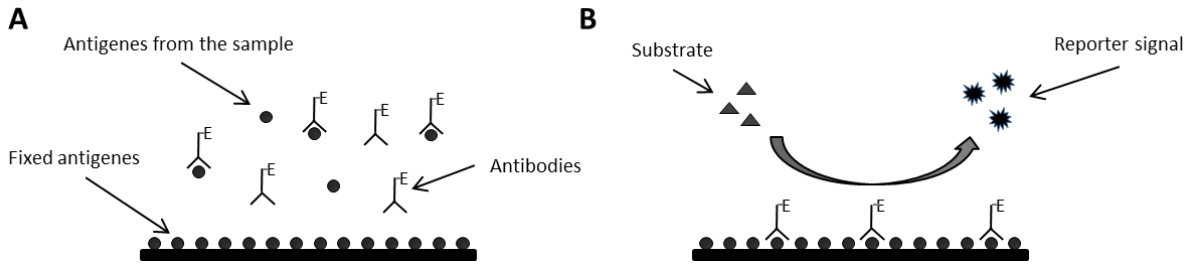

Figure 3: Principle of the competitive ELISA: Immobilized antigens and antigens from the sample compete for enzyme-linked antibodies. After a washing step to remove unbound molecules, a chromogenic substrate is added, and the resulting color intensity is indirectly proportional to the concentration of the analyte.

The microplate well is coated with specific antigens. The sample is pre-incubated with a defined amount of specific antibodies and applied to the antigen-coated plate. Thus, the bound antigens and the allergens of the food sample compete for the antibodies. After a washing step to remove unbound molecules, a chromogenic substrate is added. The color intensity catalyzed by the enzyme is measured and is indirectly proportional to the concentration of the allergen in the sample (Poms et al., 2004). In food allergen analysis, sandwich ELISAs are predominantly used (Schubert-Ullrich et al., 2009) due to their higher specificity compared to competitive ELISAs (Yeung, 2006). The principle of sandwich ELISAs is illustrated in Figure 4.

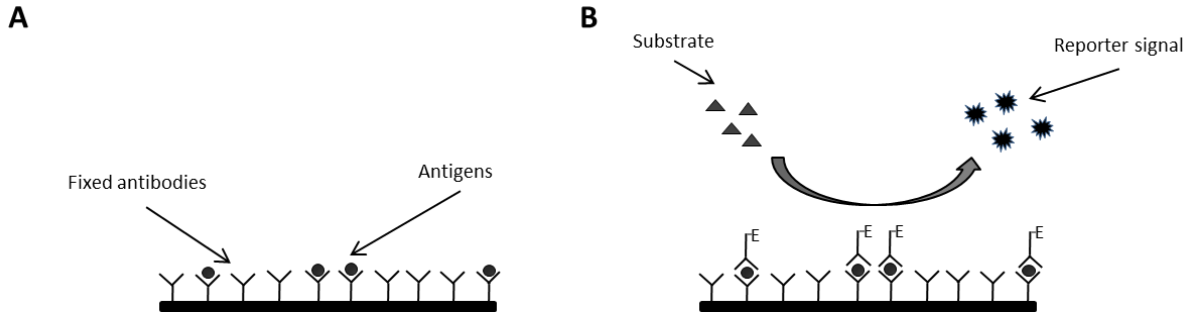

Figure 4: Principle of the sandwich ELISA: Antigens from the sample are bound to immobilized antibodies on the well. After a washing step to remove unbound molecules, a second enzyme-linked antibody is added. The resulting color intensity is directly proportional to the concentration of the analyte.

Applying a sandwich ELISA, the antigens of the sample bind to antibodies immobilized on microplate wells (contrary to the microplate well coated with antigens of the competitive ELISAs). After a washing step to remove unbound analyte molecules, the bound antigens are detected with a second, enzyme-linked antibody. The addition of

a chromogenic substrate leads to a reaction with a color change; the color's intensity is directly proportional to the analyte concentration in the sample (Poms et al., 2004).

Based on the high potential for standardization and automation of ELISAs, easy-to-use and rapid test kits have become available for a variety of food allergens. Whereas rapid ELISAs generate results within 30 to 60 minutes, the more simplified, and therefore portable, lateral-flow assays and dipsticks provide information within only a few minutes. However, the information is qualitative as long as no test strip reader is used (Schubert-Ullrich et al., 2009).

Besides the ELISA methods, MS-based methods have been developed and applied in the field of protein-based allergen detection and quantification.
For this analysis, the proteins of the food have to be extracted and purified to eliminate inhibitors. For the protein extraction mainly buffer systems are used, for purification purposes solid phase extraction (SPE), protein precipitation, ultrafiltration and size exclusion chromatography are applied. The subsequent tryptic digestion includes various steps: reduction with dithiothreitol (DTT), alkylation with indole-3-acetic acid (IAA), the actual digestion with trypsin and the acidification (e.g. with formic acid). The digested peptides are separated by liquid chromatography (LC) and analyzed by mass spectrometry (MS). The obtained MS spectra of the digested peptides are then analyzed by bioinformatic tools identifying signature peptides and thus indicating the presence of the respective allergenic substance in the analyzed food.

Allergens or rather the signature peptides are quantified by MS techniques with external calibration, standard addition or the usage of internal standard materials. The quantification can be achieved by label-free and stable isotope labeled strategies (Planque et al., 2017). Label-free are the quantification by external calibration, modified synthetic peptide approach or standard addition. External calibrations via spiking extracted proteins to the food samples are less expensive than other approaches, but require calibrations curves for each analyzed matrix. The modified synthetic peptide approach allows for a better recovery, but the addition can negatively affect retention time and ionization of the target peptide. Adding different amounts of extracted proteins to the sample before the digestion allows for considering matrix and digestion effects on the quantification by standard addition. Stable isotope-labeled quantification is based on the use of isotope-labeled proteins or peptides. The addition of a labeled

protein to the sample before the analysis allows for accurate and reliable quantitative results as the effects of protein extraction, digestion and purification can be taken into account. However, this method is very expensive and thus the applicability in routine analysis is questionable. Less expensive is the use of labeled peptides that are added after digestion before purification. Thus, matrix effects can be considered, but no negative influences during extraction and digestion (Planque et al., 2017).

3.3.2 DNA-based methods

DNA-based detection methods are characterized by the detection of specific DNA sequences coding for the allergenic protein, or other DNA sequences specific for the allergenic food of interest (EFSA, 2004). The specific DNA fragment is amplified and detected in a subsequent step. These methods can be considered complementary to the ELISA methods, especially when the analysis of proteins fails (e.g. due to foods containing low amounts of protein and processed foods with extensive modification of native proteins) (EFSA, 2004). DNA-based methods are highly specific and sensitive, and DNA is able to withstand harsh processing conditions better than proteins (Mustorp et al., 2008). However, DNA-based tests are reliant on the efficiency of DNA extraction, the degree of DNA degradation (Boye et al., 2012) and negative influences of the food matrix (e.g. co-extracted inhibiting components). PCR methods can be divided into three approaches: PCR followed by gel electrophoresis, ELISA-PCR and real-time PCR. PCR is based on the ability of the enzyme DNA polymerase to synthesize a new DNA strand complementary to the original single strand. The DNA polymerase adds free nucleotides to a pre-existing 3'-end of a DNA strand and therefore needs an oligonucleotide (primer) to start with the amplification complementary to a DNA single strand.

The PCR program consists of a number of amplification cycles. Each cycle is characterized by three functional steps that are determined by specific temperature-time-profiles (Poms et al., 2004). Initially, the temperature is increased to about 95°C for 30 – 60 s ("denaturation") and thus the double stranded DNA is melted to single strands. In the following step, the temperature is lowered to 45 – 65°C for 30 – 60 s and two oligonucleotides, the so-called forward and reverse primer, anneal complementarily to the respective single strand ("annealing"). The optimum annealing temperature of the primers needs to be considered and depends on the lengths and the composition of the primer pair. Finally, the DNA polymerase extends the primers

at its optimal working temperature of 72°C by adding, complementary to the original single strand, nucleotides to obtain a double strand (“elongation”) (Konrad, 2010). Typically, this three-step process is repeated for 25-45 cycles (Poms et al., 2004). In theory, the number of DNA fragments doubles in each cycle. The amplified product can be loaded onto an agarose gel and be visualized by staining with an intercalating fluorescent dye. Results obtained from PCR with subsequent gel electrophoresis detection are qualitative.

By contrast, quantitative results can be achieved by PCR-ELISA or real-time PCR. PCR coupled to ELISA involves the amplification of a specific DNA fragment. Then, a probe that is labeled with a protein is linked to the amplification product. An enzyme-labeled antibody binds to this protein. The quantification of the DNA concentration is performed by means of the color reaction of an enzyme-substrate reaction and a standard curve.

The real-time PCR is an advancement of the PCR using fluorescence dyes, whereas the amplification of the DNA fragments can be monitored in real-time, gel-free and quantitatively. Intercalating fluorescence dyes interact with double stranded DNA, and therefore with the amplification products, so that the fluorescence intensity of the dye increases with the amount of double stranded DNA. The usage of fluorescent-labeled DNA probes has the advantage of a sequence-specific detection, because these probes hybridize only to their complementary single strand DNA. A reporter dye and a quencher dye are attached to these target-specific hybridization probes. The probe anneals to the single stranded segment between the two bound primers (Poms et al., 2004).

The principle of real-time PCR with hybridization probes is based on fluorescence resonance energy transfer (FRET). The energy of an excited fluorescent molecule (the reporter dye) is transferred to a second fluorescent molecule (the quencher dye) when both are in spatial proximity, resulting in the quenching of the fluorescence of the reporter dye (Konrad, 2010). Amplification leads to the cleavage of the probe by the 5’-exonuclease activity of the DNA polymerase. Thus, the two dyes are separated from each other; the fluorescence is no longer suppressed by the quencher dye and can be measured. The increasing fluorescence due to the amplification is proportional to the amount of PCR product (Poms et al., 2004). The cycle number required until the fluorescence intensity exceeds the background signal corresponds to the cycle threshold (C_t). The C_t values are used for quantification (Poms et al., 2004). Serially

diluted DNA extracts with known concentrations are needed for the creation of a standard curve. Thus, the initial copy number of unknown samples can be determined. Differently labeled probes can be used to detect several PCR targets simultaneously and therefore enable the development of multiplex real-time PCR systems.

3.4 Real-time PCR-based quantification approaches

The real-time PCR results in quantitative data, the C_t values, which depend on the initial copy number of the respective target sequence. Different approaches have been developed to correlate the C_t values or rather the initial copy number and the actual amount of the respective allergenic food in the sample material.

3.4.1 Determination of DNA proportions

The quantification of allergenic foods can be achieved by the determination of DNA proportions through the calculation of the ratio of the DNA of the allergenic food to the total DNA. The amount of the DNA of the allergenic food is determined by real-time PCR. A dilution series of genomic DNA of the analyte is compared to the amount of amplifiable target DNA in order to determine the amount of target DNA in the DNA extract of the sample material. The total DNA of the extracted sample material is most commonly quantified by photometric or fluorimetric measurement. Using the determination of DNA proportions, lupine flour was quantified in wheat flour, and its applicability was shown by analysis of commercial samples and the comparison with their lists of ingredients (Scarafoni et al., 2009). However, the applicability of this method is limited to a specific food matrix. Food matrices with a lot of extractable DNA (e.g. fish or meat) or matrices with little or none extractable DNA (yogurt or seasoned salt) result in completely different DNA proportions, even though the amount of allergenic ingredient is the same. In order to obtain reliable information on the allergen content, it is necessary to establish the correlation between the DNA proportions and the actual amount of analyte for each kind of food matrix.

3.4.2 Competitive real-time PCR

Competitive DNA calibrators are synthetic oligonucleotides. These oligonucleotides are designed in a way that they compete with the probe of the specific real-time PCR system, but are detected with a distinguishable fluorescence dye. The DNA obtained by an extraction procedure is normalized: the DNA concentration is adjusted to the identical level for all examined samples through dilution. Subsequently, the DNA calibrators are added to the normalized DNA in a definite amount that corresponds to a specific allergen content. The calibrator and the probe specific for DNA from the allergenic food are detected with a duplex real-time PCR system sharing the same

primers. The resulting C_t values are compared. If the C_t value of the calibrator is higher than the respective C_t value of the specific probe, the examined sample contains more analyte than calibrator and vice versa. Holzhauser et al. (2009) calibrated a peanut-specific PCR system with a calibrator to a fictive threshold of 0.01% peanut in chocolate. This method leads to semi-quantitative results in terms of greater or less than the threshold that the added calibrator corresponds to. Therefore, the method is suitable for the surveillance of potential future thresholds, but lacks information about the order of magnitude of the allergen content. In the particular case that both real-time PCR systems show identical amplification efficiency, contents of peanut that differ from the fictive threshold can be calculated. The method's capability to be extended to other food matrices has been shown for cookie dough, ice cream, cookies and coconut muesli (Holzhauser et al., 2014). Applying this method, two competitive real-time PCR assays for the determination of peanut and soy bean have been reported (Ladenburger et al., 2018). By targeting mitochondrial DNA sequences the sensitivity of the assays could be improved and the quantification could be achieved in a range of 1 and 100 mg/kg in calibrated food matrix standards.

3.4.3 Matrix-adapted quantification

The matrix-adapted quantification uses a model matrix with a known amount of the analyte and assumes that the model matrix and the sample matrix have the same influence on the analysis. The DNA of model matrices with various amounts of analyte and the sample material are extracted and analyzed by real-time PCR. An external calibration curve is established using the results from the model matrix, and the analyte content in the sample is calculated based on this external calibration curve (Lopez, 2008; Siegel et al., 2012). This quantification approach is also the basis of commercially available kits (R-Biopharm, 2018a, 2018b, 2018c, 2018d and 2019a) in combination with an external calibrator (R-Biopharm, 2019b).

3.4.4 Internal standard material

Another possibility for the quantification is the use of a reference gene that is present in all foods in a consistent quantity. With real-time PCR systems specific for the reference gene and for the analyte, the respective copy numbers can be determined. This method is used for the quantification of genetically modified organisms (GMOs) by determining the copy number of the genetically modified DNA sequence in soy

versus the copy number of a soy bean reference gene (Wurz et al., 1999). The ratio of the copy numbers represents the quantity of analyte per amount of food. Such a reference gene is for example the gene encoding for *Myostatin* that has been used as reference gene “meat” for the differentiation of animal species in meat products (Laube et al., 2007). So far, no universal reference gene for food in general is known, especially not for complex foods. To overcome this fact, the quantification using an internal standard material depends on the addition of a standard material that does not occur naturally in food and which can be detected by means of real-time PCR. A defined amount of the standard material is added to the analyte and both, the standard material and the analyte, are analyzed by real-time PCR. With these results, a coefficient-value is calculated by dividing the initial copy number of the standard material by that of the analyte. For the quantification, the same amount of standard material is added to the sample material and co-analyzed with the analyte. The content of the analyte is then calculated by dividing the initial copy number of the analyte by the initial copy number of the internal standard and the result is multiplied with the coefficient-value and the factor 1.000.000 to obtain the result in mg allergen per kg food (Hirao et al., 2006).

3.4.5 Standard addition

The standard addition is inferred from chemical analyses and was applied in combination with real-time PCR for the first time in 2010 (Eugster, 2010). Different known amounts of the analyte are added to individual portions of the sample material and analyzed. From the resulting C_t values and the added amounts of the analyte, a curve is plotted. The shape of this curve depends on the initial content of the analyte in the sample material; the higher the analyte content is, the flatter are the resulting curves (Figure 5).

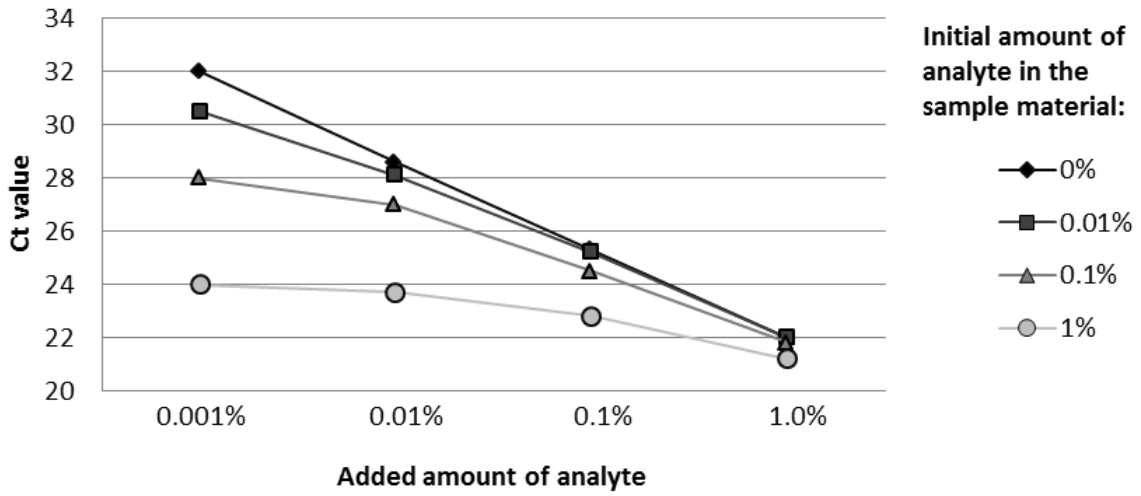

Figure 5: Dependence of the C_t values on the added initial amounts of analyte (0.001, 0.01, 0.1 and 1%) in the sample material (0, 0.01, 0.1 and 1%).

This principle can be used to determine the initial amount of the analyte in the sample material. When the efficiency of the PCR reaction is known, the C_t value can be calculated depending on the concentration of the analyte composed of initial and added analyte in the sample material with equation (I). As the added amounts for the standard addition procedure are known and all other parameters are constant, a family of curves can be determined by varying the values for the initial analyte amount.

The four experimentally determined C_t values, resulting from the standard addition procedure, are plotted to obtain the experimental curve. This curve is compared to the family of curves calculated from different initial analyte amounts. The best fitting calculated curve corresponds to the initial analyte amount in the analyzed sample material.

$$Ct = \frac{logK - log[c(A) + c(st)]}{log\left(\frac{Eff}{100} - 1\right)} \qquad \text{(I)}$$

C_t	*cycle threshold*
K	*1/B; whereas B is a constant, determined with an efficiency of 90%*
and	*a concentration of the analyte of 100%*
c(A)	*initial concentration of the analyte in the sample material*
c(st)	*added concentration of the analyte as part of the standard addition*
Eff	*efficiency of the real-time PCR system*

The best fitting curve is ascertained by approximate computation assuming various initial analyte contents and calculating the C_t values for all four added analyte concentrations (1%, 0.1%, 0.01% and 0.001%) according to equation (I). The following 5 steps describe the procedure to determine the actual amount of the analyte in the sample material:

1. The experimentally determined C_t value of the highest added analyte amount (1%) is subtracted from the respective calculated C_t value, resulting in the ΔC_t value (Figure 6).

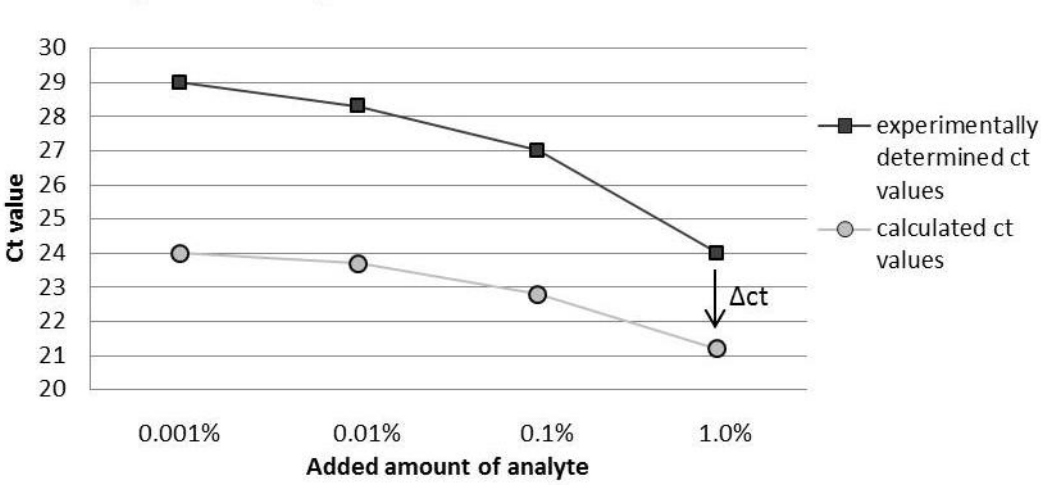

Figure 6: First step of the standard addition method to calculate the initial analyte amount; the ΔC_t value is calculated by subtracting the experimentally determined C_t value of the highest added analyte amount (1%) from the respective calculated C_t value.

2. This ΔC_t value is subtracted from all four experimentally determined C_t values (Figure 7).

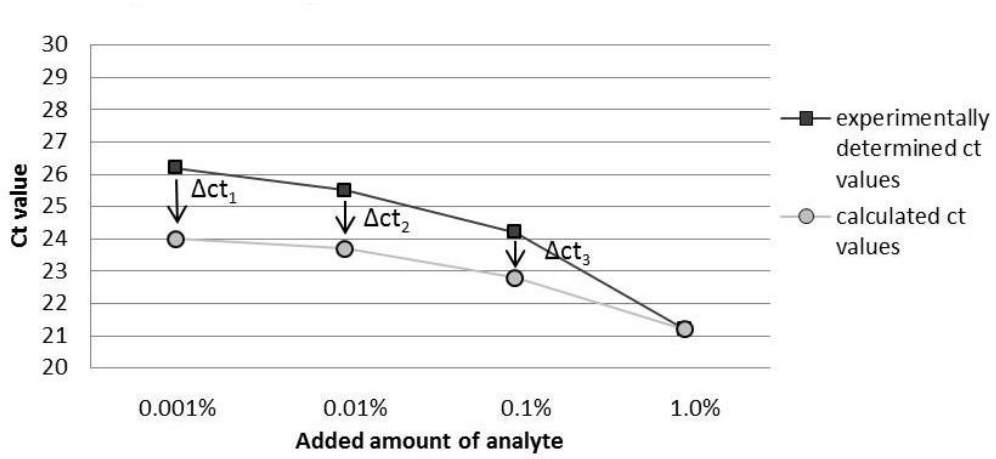

Figure 7: Second step of the standard addition method to calculate the initial amount of analyte; after subtracting ΔC_t value from all four experimentally determined C_t values, the error sum of squares from the three obtained ΔC_t values (ΔC_{t1}, ΔC_{t2} and ΔC_{t3}) are determined.

3. The error sum of squares (ESS) from the three obtained ΔC_t values (ΔC_{t1}, ΔC_{t2} and ΔC_{t3}) is determined with equation (II).

$$ESS = [(x + C_t(0.001\%)_{determined}) - (x + C_t(0.001\%)_{calculated})]^2 + [(x + C_t(0.01\%)_{determined}) - (x + C_t(0.01\%)_{calculated})]^2 + [(x + C_t(0.1\%)_{determined}) - (x + C_t(0.1\%)_{calculated})]^2 \quad \text{(II)}$$

ESS	*error sum of squares*
x	*unknown analyte concentration of the examined sample material*
C_t*(0.001%)*$_{determined}$	C_t *value of the analyte concentration 0.001% experimentally determined*
C_t*(0.001%)*$_{calculated}$	C_t *value of the analyte concentration 0.001% calculated etc.*

4. A different initial concentration of the analyte (c_A) is assumed and the ESS is re-calculated.

5. Steps 1 to 4 are repeated until the ESS reaches its minimum. The assumed initial concentration of the analyte (c_A) is then presumed to be equal to the actual amount of analyte in the sample material.

4 Results

4.1 Publication I

Florian Luber, Anja Demmel, Denise Herbert, Anne Hosken, Christine Hupfer, Ingrid Huber, Ulrich Busch, Karl-Heinz Engel

Comparative assessment of DNA-based approaches for the quantification of food allergens.

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Summary

Food allergies are a common health concern and affect about 3 to 6% of the population. Governments implement labeling regulations to offer sensitive consumer the possibility of the avoidance of the respective food. To be able to monitor the surveillance of these regulations, reliable analytical methods are required. Different strategies for quantification purposes were selected: a matrix-adapted calibration, a quantification using an internal standard material and a standard addition method. To be able to compare the performances of these different DNA-based approaches, uniform experimental conditions were defined. Spiked and homogenous boiled sausage material was applied for the validation to be able to determine pre-defined performance parameters. The determination of the recovery, the precision, expressed by the repeatability and the reproducibility, and the sensitivity (LOD and LOQ) allows for a comparative assessment of the different quantification strategies. The evaluation of the validation data revealed benefits and limitations of the different methods, and the standard addition although laborious emerged as the most promising method based on the performance criteria. The applicability of the methods for the analysis of routine samples was tested using self-prepared spiked dough and muffins baked thereof.

Candidate's contribution

Literature-based selection of quantification strategies to be tested; preparation of reference materials and model food matrices; definition of uniform experimental framework conditions; setting up of uniform assessment criteria; evaluation of the results and statistical assessment; interpretation of the data set; drawing up and compilation of Figures and Tables; writing of the manuscript; revision of the manuscript.

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Analytical Methods

Comparative assessment of DNA-based approaches for the quantification of food allergens

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ABSTRACT

Governments all over the world have implemented regulatory frameworks concerning food allergen labelling and established or discussed the implementation of thresholds. Therefore, quantitative methods are needed for their surveillance. DNA-based approaches using a matrix-adapted calibration, an internal standard material and a modified standard addition have been developed. In order to enable a comparative assessment of the available quantification methods, experimental framework conditions and uniform performance criteria were defined. For the evaluation of the experimental results using homogenous sample material, the recovery, repeatability and reproducibility were considered along with the limit of detection and the limit of quantification. In addition, muffin dough and muffins spiked with sesame were analysed to assess the suitability of the methods to quantify sesame in model foods. The modified standard addition emerged from the comparative assessment and the analysis of the model foods to be the most appropriate method to quantify traces of allergens in food.

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1. Introduction

In Western countries, food allergies affect approximately 5% of young children and 3–4% of adults (Sicherer & Sampson, 2010). Although several strategies with the aim of long-term treatment and possible cure for established food allergies are being discussed, the current standard of care is the avoidance of food allergens (Wang & Sampson, 2012). To offer the consumers the opportunity to avoid foods that might lead to adverse immunologic responses, governments all over the world have implemented regulatory frameworks concerning the labelling of food allergens (Gendel, 2012). In the European Union, the allergenic food ingredients, for which labelling is mandatory are listed in Annex II of the Regulation 1169/2011/EC on the provision of food information to consumers (European Parliament & European Council, 2011). However, this regulation does not address allergens that are unintentionally present owing to cross-contact in the course of the manufacturing process. Regulation 2002/178/EC (European Parliament & European Council, 2002) states that food shall not be placed on the market if it is unsafe. Even traces of food allergens can lead to several allergenic reactions and represent a health risk, especially if they are not mentioned on the label. Due to their duty of care, food manufacturers often use "may contain" statements. This precautionary labelling has negatively affected the confidence of allergenic consumers in the safety of foods (Kerbach et al., 2009). The definition of thresholds, above which labelling of a food allergen is mandatory, would be an appropriate approach to overcome the uncertainty of the consumers. To monitor the compliance with these thresholds, both food industry and food safety authorities need methods for the quantitative analysis of food allergens.

Immunochemical methods, mainly ELISAs, are widely used to quantify food allergens (Kerbach et al., 2009). Recently, mass spectrometry-based methods have been developed and may offer alternative methods in the future (Heick, Fischer, & Pöpping, 2011). In addition, DNA-based methods are used mainly as screening tools to detect the presence of allergenic foods/components. The real-time PCR-based methods result in quantitative data, the ct-values, which depend on the initial copy number of the respective target sequence. Although the relationship between copy number and content of allergenic protein is not fixed, correlations between the ct-values and the contents/amounts of allergenic foods or their components can be established. Therefore, different quantification

Abbreviations: Bp, base pair; Ct, cycle threshold; ELISA, enzyme-linked immunesorbent assay; GMO, genetically modified organism; LOD, limit of detection; LOQ, limit of quantification; NTC, no template control; PCR, polymerase chain reaction.

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methods using real-time PCR have been developed; they are based on three principles: The matrix-adapted quantification depends on an external calibration using a (model-)matrix similar to the sample matrix and spiked with the analyte of interest. Another quantification method is based on the addition of an internal standard material that is co-analysed. The actual analyte content is then calculated from the ratio of the copy numbers (analyte and internal standard material) in the sample material and in a so-called coefficient-value sample (Hirao, Hiramoto, Imai, & Kato, 2006). A third approach to quantify allergenic foods is the modified standard addition. The analyte is added to individual portions of the sample in different concentrations, DNA is extracted from these sample portions and analysed by means of real-time PCR. The concentrations of the added analyte are plotted against the resulting ct-values. The shape of the resulting curve is characteristic for the initial analyte content of the sample, which can be calculated by comparing the shape of the curve with curve shapes of known analyte concentrations (Eugster, 2010).

Due to the lack of reference materials and standardised validation protocols for DNA-based quantification methods for allergenic foods, a comparison of the performances of the different quantification methods was not possible so far. Therefore, the objective of this study was to set up experimental framework conditions that allow a comparative assessment of the different quantification approaches. Materials tested for homogeneity were analysed using three quantification methods and the results were evaluated on the basis of uniform assessment criteria. In addition, the suitability of the methods to quantify sesame in model foods was investigated by analysis of muffin dough spiked with different amounts of sesame and of the respective baked muffins.

2. Materials and methods

2.1. Materials

2.1.1. Sample material

Unpeeled sesame seeds were obtained from Seeberger (Ulm, Germany), *Gypsophila elegans* seeds, used as internal standard material, were from Samenzucht Hoffmann (Forchheim, Germany). Both materials were ground with a hand blender (M160, ESGE, Mettlen, Switzerland) before use. Breadcrumbs were purchased in a local store and tested for the absence of sesame DNA by means of real-time PCR. The breadcrumbs were mixed with the ground sesame seeds in concentrations of 100,000, 10,000, 1000, 100, 10 mg/kg, using a hand blender (M160, ESGE, Mettlen, Switzerland).

The sample material "boiled sausages" was provided by the Chemisches und Veterinäruntersuchungsamt, Freiburg, Germany. This material had been spiked with sesame in concentrations of 5, 10, 20 and 100 mg/kg and tested for homogeneity (Siegel, Schnur, Boernsen, Pietsch, & Waiblinger, 2012).

Muffin dough was prepared using cooked chickpeas (1112 g), water (309 g), wheat flour (810 g), baking powder (65 g), iodized salt (31 g), eggs (463 g), rapeseed oil (350 g), Ajvar (77% paprika, 4% sunflower oil, 12.5% eggplant, sugar, salt, garlic, brandy vinegar and 1% pepperoni; 353 g) and mixed herbs (cinnamon, fennel, star anise, pepper; 4 g) and was homogenized using a mill (Retsch, Haan, Germany). A batch of the dough was analysed by means of real-time PCR to identify possible cross reactions of the matrix or a contamination with sesame. To obtain samples with the concentration of 10,000 mg/kg sesame, 3 g of sesame paste was added to 297 g dough and homogenized with a thermo mixer (Vorwerk, Wuppertal Germany). After homogenization, the spiked dough was mixed with sesame-free dough in subsequent steps to receive muffin doughs containing 100, 20, 10 and 5 mg/kg sesame, respectively.

Half of the dough was baked as muffins in a cabinet dryer (Memmert, Schwabach, Germany) at 200 °C for 16 min. To avoid contamination, the doughs corresponding to the different dilution levels were baked separately. For calculation of the water loss, each muffin was weighed before and after the baking procedure (Kern, Balingen-Frommern, Germany). The samples were ground with a hand blender (M160, ESGE, Mettlen, Switzerland) and stored at −20 °C until use.

2.1.2. Oligonucleotides

All primers and probes (Table 1) were produced by TIB MOLBIOL (Berlin, Germany). The sesame system published by Mustorp, Engdahl-Axelsson, Svensson, and Holck (2008) detects the gene coding for the storage protein 2S-albumin of *Sesamum indicum* (Ses i 1). The target of the inhibition control system is an artificial random sequence of 145 bp that is embedded in the plasmid ICall (Table 2). The anthocyanidine-synthase gene (AY256380) is targeted by the *G. elegans* PCR system. For the determination of the exact copy number of the respective system, the plasmid SesGyp was designed that contains the system specific target sequences of sesame and *G. elegans* (Table 2). The specific target sequences are separated by a random sequence of 35 bp. The plasmids were obtained from Eurofins MWG Operon (Ebersberg, Germany).

2.1.3. Commercial sesame quantification kits

For the quantification of sesame protein in the muffin dough and in the baked muffins, a commercial sandwich ELISA kit (Biokits, Sesame Assay Kit 902070X) from NEOGEN (Lansing, MI, USA) was used. The sesame concentration of the sample materials is calculated using a calibration curve from a protein standard solution contained in the kit.

2.2. Methods

2.2.1. Matrix-adapted quantification

The matrix-adapted quantification uses a model matrix with a known amount of the analyte and assumes that the model matrix and the sample matrix have the same influence on the analytics. An external calibration curve is established using the results of the analysis of the model matrix containing a known amount of the analyte, and the analyte content in the sample is calculated based on the external calibration curve (Siegel et al., 2012).

The DNA was extracted following a CTAB protocol. The sample material was homogenized using a hand blender (M160, ESGE, Mettlen, Switzerland); 2 g were weighed into a 50 mL falcon tube and incubated over night at 65 °C with 10 mL of CTAB buffer [2% (w/v) cetyltrimethylammoniumbromide, 1.4 M NaCl, 0.1 M 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), and 20 nM ethylenediaminetetraacetic acid (EDTA) at pH 8] and 30 µL Proteinase K (>600 mAU/mL) (Qiagen, Hilden, Germany) in a platform shaker with incubator (Heidolph Instruments, Schwabach, Germany). After centrifugation at 7200*g* for 10 min, 1000 µL of the supernatant was added to 700 µL ReadyRed chlorophorm/isoamylalcohol (MP Biomedicals, Illkirch, France) in a 2 mL tube and shaken in an overhead shaker (Heidolph Instruments, Kehlheim, Germany) for 15 min. The sample was centrifuged for 15 min at 20,000*g* and 750 µL of the clear supernatant was added to 750 µL of cold isopropanol and incubated at room temperature for 30 min. The sample was centrifuged at 20,000*g* for 15 min, the supernatant was discarded and 500 µL of ethanol (70%) was added. After gentle shaking, the sample was centrifuged at 20,000*g* for 5 min, the ethanol was removed and the residual pellet was dissolved in TE buffer (1×) [10 mM TRIS × HCl at pH ~8.0, containing 1 mM EDTA]. The dissolved DNA was purified using the QiaQuick PCR purification kit (Qiagen, Hilden Germany) and eluted with 30 µL elution buffer.

Table 1
Oligonucleotides for the detection of DNA from sesame, *Gypsophila elegans* and the inhibition control system.

Name	Function	Sequence	Length	Target accession number
Ses F	Primer	5′-CCAGAGGGCTAGGGACCTTC-3′	20 bp	AF240005
Ses R	Primer	5′-CTCGGAATTGGCATTGCTG-3′	19 bp	
Ses S	Probe	5′-6FAM-TCGCAGGTGCAACATGCGACC–TMR-3′	21 bp	
Gyp F	Primer	5′-GAAATGGTTATTGCCACGAAAGT-3′	23 bp	AY256380
Gyp R	Primer	5′-GCCTCACATACTCCTTAGGAATGC-3′	24 bp	
Gyp S	Probe	5′-YAK-CGATTCCACTACTTGCTAGGGTCTCAAC–BBQ-3′	28 bp	
ICall F	Primer	5′-AACCCCAGACGGCTGT GAAT-3′	20 bp	Random sequence
ICall R	Primer	5′-AATACTGCCCCAGAGTG GCT-3′	20 bp	
ICall S	Probe	5′-ROX-ACAGTGGCAGTCT TATTGCATCTCGCC–BBQ-3′	27 bp	

Table 2
Inserted nucleotide sequences of the plasmids.

Name	Nucleotide sequence	Length
ICall	5′-GCTACG*AAACCCCAGACGGCTGTGAATT***ACAGTGGCAGTCTTATTGCATCTCGCC**AACTGTTAA TCT*AGCCACTCTGGGGCAGTATTT*CCTAAGATGATATCCTCCAGGCGCAAGCCCAAGGGCCGGACCTTCAACGTTACCCGT-3′ Italics: Position of the primers (ICall F and ICall R) Bold: Position of the probe (ICall P)	145 bp
SesGyp	5′-*CCAGAGGGCTAGGGACCTTCC***TCGCAGGTGCAACATGCGACC***CCAGCAATGCCAATTCCGAC*TAA GATCCGTGAGGGACGCGACAGAGCCCTACGAC*gaaatggttattgccacgaaagt*CGCTCCCTCCAGG**gttgagaccctagcaagtagtggaatcg** AAT*gcattcctaaggagtatgtgaggc*-3′ Italic upper case letters: position of the primers (Ses F and Ses R) Bold upper case letters: position of the probe (Ses P) Highlighted in grey: random nucleotide sequence Italic lower case letters: position of the primers: (Gyp F and Gyp R) Bold lower case letters: position of the probe (Gyp P)	188 bp

The DNA concentration was determined using the fluorometrical intercalating dye Quant-iT PicoGreen dsDNA reagent (Invitrogen, Karlsruhe, Germany) on a GENious plus reader (Tecan, Männedorf, Switzerland). λ-DNA (Invitrogen, Karlsruhe, Germany) was used to generate a calibration curve. The DNA concentration of the samples was calculated using the obtained linear equation. The purified DNA extracts were normalised to a concentration of 20 ng/μL and stored at −20 °C until they are used.

In the duplex real-time PCR, 5 μL of the extracted DNA was used as template and added to 20 μL of reaction mix. The composition of the reaction mix was as follows: QuantiTec Multiplex PCR NoROX Mastermix (1×) (Qiagen, Hilden, Germany), forward primer sesame (0.2 μM), reverse primer sesame (0.2 μM), and probe sesame (0.1 μM); forward primer ICall (0.2 μM), reverse primer ICall (0.2 μM), and probe ICall (0.1 μM); and plasmid ICall (500 plasmids per reaction). The duplex real-time PCR was performed in a Stratagene MxPro 3005 real-time cycler (Agilent Technologies, Loveland, CO, USA) starting with an initial activation of the polymerase at 95 °C for 10 min, followed by 45 cycles of denaturation (95 °C, 15 s) and annealing/elongation (60 °C, 1 min). The thresholds were set to the beginning of the exponential phase of the amplification curves as determined by visual examination, for the FAM dye at 500 fluorescence units and for the ROX dye at 400 fluorescence units. The ct-values were generated with the MxPro software (V4.10, Agilent Technologies, Loveland, CO, USA) and used for the calculation of the respective sesame contents.

The DNA from the sesame/breadcrumbs matrix (10–100.000 mg/kg) was extracted three times, purified, normalised to 20 ng/μL and analysed in triplicate using the duplex real-time PCR. The means of the ct-values were plotted against the corresponding sesame amounts to generate an external standard curve.

Assuming that the mean of the inhibition control system is consistent if no inhibition occurs in the PCR, the mean ct-value of the inhibition control PCR was used to assess the quality of the respective real-time PCR runs. The mean of the ct-values of the inhibition control system was calculated for each single real-time PCR run. In addition, the ct-values of the inhibition system of the run containing the calibration curve were averaged. The ratio of both mean ct-values is drawn and the resulting factor was used to normalise the ct-values that were used to calculate the sesame contents. Through the normalization with this factor, the standard curve does not need to be determined in every run, but can be generated in advance.

2.2.2. Quantification using an internal standard material

The quantification using an internal standard material depends on the addition of a standard material that does not occur naturally in the food. A defined amount of the standard material is weighed to the analyte and analysed by means of real-time PCR. With these results, a coefficient-value is calculated through the division of the initial copy number of the standard material by that of the analyte. For the quantification, the same amount of standard material is weighed to the sample material and co-analysed with the analyte. The content of the analyte is then calculated by dividing the initial copy number of the analyte by the initial copy number of the internal standard multiplying the result with the coefficient value and the factor 10^6 as described by Hirao et al. (2006).

G. elegans seeds (200 mg) and homogenized sample material (1000 mg) were weighed into a 50 mL falcon tube. The DNA was extracted, purified and normalised following the protocol described in Section 2.2.1. The extracted DNA (5 μL) was used as template in the triplex real-time PCR with 20 μL of reaction mix. The composition of the reaction mix was as follows: QuantiTec Multiplex PCR NoROX Mastermix (1×) (Qiagen, Hilden, Germany), forward primer sesame (0.2 μM), reverse primer sesame (0.2 μM), and probe sesame (0.1 μM); forward primer ICall (0.2 μM), reverse primer ICall (0.2 μM), and probe ICall (0.1 μM); plasmid ICall (500 plasmids per reaction); forward primer Gyp. e. (0.3 μM), reverse primer Gyp. e. (0.3 μM) and probe Gyp. e. (0.2 μM). The triplex real-time PCR was performed using the same conditions as described in Section 2.2.1 for the duplex real-time PCR. The thresholds were set to the beginning of the exponential phase of the

amplification curves as determined by visual examination; 500 fluorescence units for the FAM dye, 400 fluorescence units for the ROX dye and 200 fluorescence units for the HEX dye. The ct-values were generated with the MxPro software and used for the calculation of the respective sesame contents of the samples.

To calculate the concentration of sesame in the samples in mg/kg, a coefficient-value was required. To obtain this value, 1000 mg of sesame seeds was mixed with 0.200 g of *G. elegans* seeds and the DNA was extracted from the mixture. This procedure was repeated 14 times (15 times in total). The coefficient-value samples were tested threefold with the triplex real-time PCR. For the calculation of the initial copy numbers, the plasmid SesGyp (Eurofins MWG Operon, Ebersberg, Germany) was used to generate two standard curves, one for sesame and one for *G. elegans*, with dilutions of 1,000,000, 500,000, 200,000, 100,000, 50,000, 10,000, 1000, 100, 50, 20, 10, and 5 copies per PCR. To obtain the coefficient value, the mean copy number of *G. elegans* was divided by the mean copy number of sesame.

The model food (1000 mg) was mixed with 200 mg of *G. elegans* seeds and extracted. The samples were measured by means of triplex real-time PCR. The plasmid SesGyp was used for creating two standard curves, one for sesame and one for *G. elegans*, comprising 12 different dilution steps (100000, 50,000, 10,000, 1000, 100, 50, 20, 10, 5, 2, 1 and 0.1 copies per PCR). The two standard curves were used for determining the respective initial copy numbers. The sesame content in mg/kg is calculated multiplying the coefficient-value and the ratio of the initial copy numbers of sesame and *G. elegans* received from the samples and multiplied with 100,000 (Hirao et al., 2006).

2.2.3. Quantification by modified standard addition

The modified standard addition is inferred from chemical analytics. Known amounts of the analyte are added to individual portions of the sample material and analysed. From the results (ct-values) and the added amounts of the analyte, a curve is plotted. The shape of this curve depends on the initial content of the analyte in the sample material. By comparing the shape of the experimental curve with the shapes of curves calculated using different initial analyte amounts, the actual content of the analyte can be determined (Eugster, 2010).

The homogenized sample material (1000 mg) was weighed into a 50 mL falcon tube. For the lysis, 5 mL CTAB buffer and 15 μL proteinase K (>600 mAU/mL) were added and incubated over night at 65 °C. In parallel, 1000 mg of the analyte was subjected to the same conditions. After the incubation, the samples and the analyte were centrifuged at 7200*g* for 10 min and the standard addition was performed. The incubated analyte was diluted with 1xTE buffer (1:100, 1:1000, 1:10,000 and 1:100,000 analyte:TE-buffer) and 40 μL of the dilutions were added to 400 μL of the incubated samples, respectively. To these mixtures, 400 μL ReadyRed chlorophorm/isoamylalcohol (MP Biomedicals, Illkirch, France) were added and shaken in an overhead shaker (Heidolph Instruments, Kehlheim, Germany) for 15 min and centrifuged at 20,000*g* for 15 min. The supernatant (375 μL) was added to 400 μL cold isopropanol and incubated for 30 min at room temperature and centrifuged again at 20,000*g* for 15 min. The isopropanol was discarded and 500 μL of ethanol were added to the residual pellet and gently shaken. The sample was centrifuged by 20,000*g* for 5 min, the pellet was dried in a vacuum centrifuge for 10 min und dissolved in 100 μL 1xTE buffer.

The DNA purification, the normalization of the DNA concentration and the real-time PCR were carried out as described in Section 2.2.1.

The quantification was performed as described by Eugster (2010) with two modifications. A calibration curve of ten-fold DNA dilutions was co-analysed to consider the variable PCR efficiencies. The second modification is the lower concentration of the added analyte for the standard addition than in the original work. The quantification using the standard addition is applied in Switzerland, where the threshold for mandatory allergen labelling is 1.0 g/kg. To be able to quantify in a lower range (5–100 mg/kg), the added analyte amounts were decreased by the factor 100–0.1%, 0.01%, 0.001% and 0.0001%.

2.2.4. Quantification using an ELISA kit

The quantification using the sandwich ELISA kit (NEOGEN, Lansing, MI, USA) was performed according to the manual provided with the kit. After the preparation of the sample extraction buffer and the enzyme immunoassay (both included in the kit), 10 g of the samples and controls were extracted and analysed in duplicate and the results were averaged. The fluorescence was measured on a GENious plus reader (Tecan, Männedorf, Switzerland). The samples were quantified using a dilution of the sesame protein standard solution (included in the kit) with a final sesame concentration of 6.25, 12.5, 25, 50 and 100 ppm.

2.3. Statistical analysis

The repeatability is described by the relative standard deviation under repeatability conditions (RSD_r). The repeatability shows the deviation of the values obtained for a reference material analysed by the same operator on two different days (2 DNA extracts, 4 PCR replicates per extract at day 1 and 2 PCR replicates per extract at day 2, respectively). The RSD_r is calculated by the ratio of the weighted standard deviation and the weighted mean expressed in percentage.

The weighted standard deviation is calculated by Eq. (I) (Hougs et al., 2011).

$$\mathrm{sd}_{\mathrm{weighted}} = \sqrt{\frac{\sum_{i=1}^{k}(n_i - 1) \times sd_i^2}{\sum_{i=1}^{k} n_i - k}} \quad \text{(I)}$$

sd, standard deviation.
n, number of replicates per extract.
k, number of values, that should be weighted.

The weighted mean is determined by Eq. (II).

$$m_{\mathrm{weighted}} = \sqrt{\frac{\sum_{i=1}^{k}(n_i - 1) \times m_i^2}{\sum_{i=1}^{k} n_i - k}} \quad \text{(II)}$$

m, mean.
n, number of replicates per extract.
k, number of values, that should be weighted.

The reproducibility is described by the relative standard deviation under reproducibility conditions (RSD_R), that is, the deviation of values obtained on two different days by two different operators using different equipment (2 DNA extracts, 4 PCR replicates at day 1 (operator 1, pipette set 1) and 2 PCR replicates at day 2 (operator 2, pipette set 2), respectively). The RSD_R is calculated by the ratio of the weighted standard deviation Eq. (I) and the weighted mean Eq. (II) and expressed in percentage.

For the description of the sensitivity of a method, expressed in terms of limit of detection and limit of quantification, a pragmatic approach is provided that refers to the uncertainty of the measurement within a 95% confidence interval (Waiblinger, Gutmann, Hädrich, & Pietsch, 2001).

The LOD is defined as the analyte concentration with a relative confidence interval $\leqslant$50% (Waiblinger, Graf, Broll, Grohmann, & Pietsch, 2011). The limit of quantification is defined as the analyte

concentration with a relative confidence interval ($P = 95\%$) $\leqslant 30\%$ (Waiblinger et al., 2001).

For the determination of the LOD and LOQ, the uncertainty of the measurement is calculated for each concentration level by Eq. (III).

$$u = \frac{t \times \mathrm{sd}}{\sqrt{n}} \quad \text{(III)}$$

u, uncertainty of the measurement.
sd, standard deviation.
n, number of replicates.
t, student factor (depending on the number of replicates n, and the confidence interval).

The relative uncertainty of the measurement is calculated by Eq. (IV) from the ratio of the uncertainty of the measurement and the respective mean.

$$u_{\mathrm{rel}} = \frac{u}{m} \quad \text{(IV)}$$

u_{rel}, relative uncertainty of the measurement.
m, mean.

The relative uncertainty is plotted against the respective concentration level of the analyte. The LOD is the intersection point of the curve with the straight line of $y = 50\%$ and the LOQ with the straight line of $y = 30\%$. The derived equation of the trend line is used for the calculation; the equation is solved for x and $y = 50\%$ for the determination of the LOD and $y = 30\%$ for of the LOQ.

3. Results and discussion

3.1. Experimental framework conditions

The comparative assessment of DNA-based quantification methods was performed using sesame as analyte and employing the real-time PCR system published by Mustorp et al. (2008) for detection. To be able to monitor possible inhibition effects on the real-time PCR due to co-extracted inhibitory factors, the inhibition control system ICall was included that detects an artificial random sequence of 80 bp embedded in a plasmid. This sequence has no homologies to any other organism as determined by Basic Local Alignment Search Tool (National Library of Medicine) (BLAST) database search, so that it should not occur naturally in food matrices. No increase of the fluorescence signal was observed testing DNA extracted from breadcrumbs, muffin dough, muffins and *G. elegans*. The absence of inhibition was proved by comparing the ct-values of the ICall system for the samples and the NTC. No inhibition was present if the ct-values of the samples were within the ct-value of the NTC ± 1.0, else, the PCR reaction was considered as inhibited.

Breadcrumbs were used as model-matrix material for the matrix-adapted quantification.

Seeds of *G. elegans* were used as internal standard material, because *G. elegans* does not occur naturally in food and is available in sufficient quality and quantity. *G. elegans* is detected by a real-time PCR system targeting a specific nucleotide sequence on the anthocyanidine synthase gene (AY256380). The standard curves needed for the determination of the respective initial copy numbers for sesame and *G. elegans* were generated by measuring ten-fold dilution series of the plasmid SesGyp.

The coefficient values of two different batches of *G. elegans* seeds were determined. The first batch of standard material (coefficient value: 0.056781) was used for the validation of the method, while the analysis of the dough and muffin material was carried out using the second batch of *G. elegans* seeds (coefficient value: 0.049525).

The standard addition method published by Eugster (2010) was performed using 0.0001%, 0.001%, 0.01% and 0.1% of the analyte diluted in CTAB-buffer and Proteinase K for the standard addition. A ten-fold dilution of the analyte was analysed and the PCR efficiency of every run was taken into account for the calculation of the quantitative results. For the chosen real-time PCR system the constant $B = 5.055 \times 10^{-8}$ was calculated using formulas 1 and 2 as described by Eugster (2010) and the ct-value for pure sesame was experimentally determined to be 19.00.

3.2. Performance criteria

3.2.1. Recovery

The recovery is defined as the compliance between a measured value of a reference material and the true value thereof. In the case of GMO (genetically modified organism) analysis, the term trueness is used instead of recovery and the acceptance criterion is a result within ±25% of the reference value (Hougs et al., 2011). For residue analysis, a widely accepted range for the recovery rate is 70–120% (Vogelgesang and Hädrich (1998)). Following the AOAC Official Methods for analysis (2012) of allergens which are based on ELISA methods, recoveries between 50% and 150% are considered acceptable as long as they are shown to be consistent (AOAC International, 2012). For the boiled sausages (5, 10, 20 and 100 mg/kg sesame), the recovery was determined using two DNA extracts per concentration level and 8 PCR replicates per extract, respectively. The results of the recovery analysis are shown in Fig. 1 for the different quantification methods. Due to the significant overestimations obtained by the quantification using an internal standard material (recoveries from 204% to 367%), none of the above mentioned criteria are met. High recoveries using this method were also observed for the quantification of lupine with *Limonium sinuatum* as internal standard material (Demmel, Hupfer, Busch, & Engel, 2012). The matrix-adapted quantification shows an extreme underestimation of 12–18% of the actual contents. This method provides precise data (small standard deviations), but depending on the applied matrix, a systematic divergence is observed. The modified standard addition results in recoveries in the range of 72–114% and thus the performance criteria recommended by Vogelgesang and Hädrich (1998) are met.

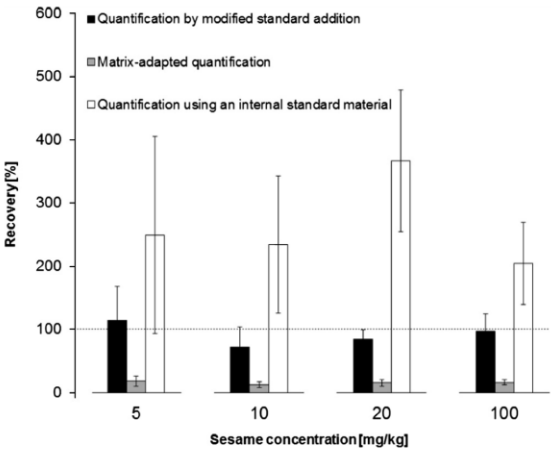

Fig. 1. Recoveries of sesame in spiked boiled sausage (adjusted sesame concentrations: 100, 20, 10 and 5 mg/kg). The dotted line represents 100% recovery.

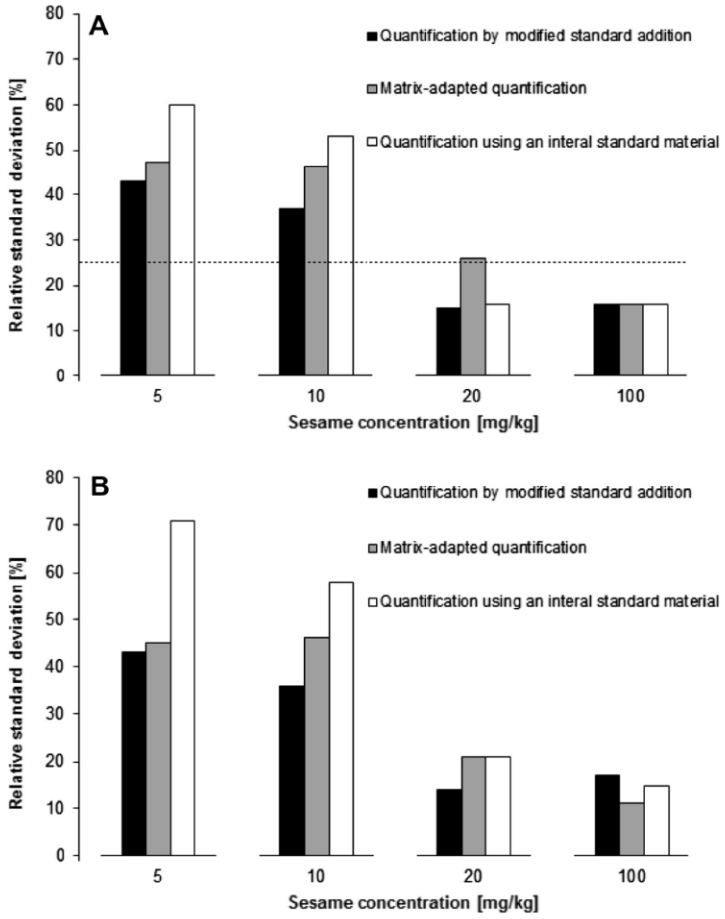

Fig. 2. Repeatability (A) and reproducibility (B) of the quantification methods for the determination of sesame in boiled sausage material with concentrations of 100, 20, 10 and 5 mg/kg. The dotted line indicates the performance criterion ($RSD_r \leqslant 25\%$) according to ENGL (Hougs et al., 2011).

3.2.2. Repeatability

The repeatability is described by the relative standard deviation under repeatability conditions (RSD_r) and is therefore a parameter for the precision of the applied method (Hougs et al., 2011). An RSD_r of ⩽25% is accepted in the fields of GMO analytics (Hougs et al., 2011). As shown in Fig. 2A, this requirement is met by all the methods employed in this studies for the sample materials containing 20 and 100 mg/kg sesame, respectively, except for the matrix-adapted quantification of the 20 mg/kg sesame containing material ($RSD_r = 26\%$). In the samples containing 5 and 10 mg/kg of sesame the RSD_r values are above the mentioned threshold for all quantification methods (Fig. 2A). However, it should be kept in mind that the GMO requirements are defined for analyte concentrations in a range of 0.1–1% (equaling 1000–10,000 mg/kg). For trace analysis of food allergens in the range of 1–1000 mg/kg, different criteria should be discussed and applied.

3.2.3. Reproducibility

The reproducibility is described by the relative standard deviation under reproducibility conditions (RSD_R); it is a parameter for the precision and additionally reflects the capacity of the applied method to remain unaffected by different operators and equipment. As shown in Fig. 2B, the RSD_R values for the boiled sausage material with sesame concentrations of 5 mg/kg range from 43% (matrix-adapted quantification) to 71% (quantification using an internal standard material). The sample material with 10 mg/kg sesame results in RSD_R values not exceeding 58% (quantification using an internal standard material). In the sample materials containing 20 and 100 mg/kg, respectively, the RSD_R values are within a range of 11–21% for all the applied methods. The RSD values for the repeatability and the reproducibility are similar for the different concentration levels and, therefore, the applied methods remain unaffected by two different operators.

3.2.4. LOD and LOQ

The limit of detection is the concentration of an analyte that can be distinguished from the blank value. In the case of GMO analysis, a pragmatic approach is provided that refers to the uncertainty of the measurement within a 95% confidence interval (Waiblinger et al., 2001). The LOD is defined as the analyte concentration where the relative uncertainty of a measurement for relative confidence interval ($P = 95$) is ⩽50% (Waiblinger et al., 2011). The LOD of the matrix-adapted quantification was determined to be 0.2, 2.4 mg/kg for the quantification using an internal standard material and 0.1 mg/kg for the quantification with the modified standard addition.

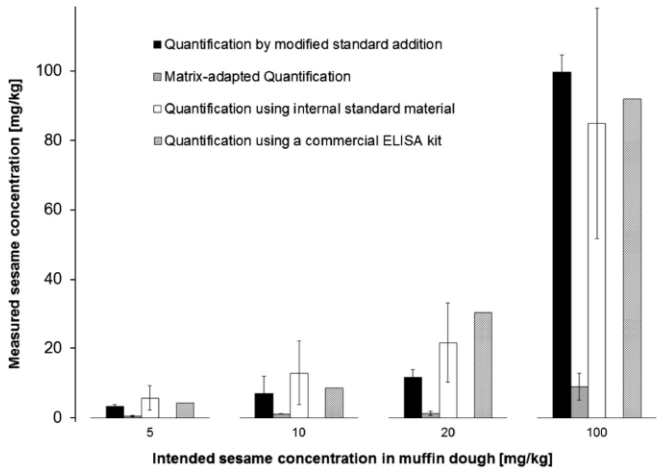

Fig. 3. Comparison of the quantification of sesame in self-prepared muffin dough samples with adjusted concentrations of 100, 20, 10 and 5 mg/kg.

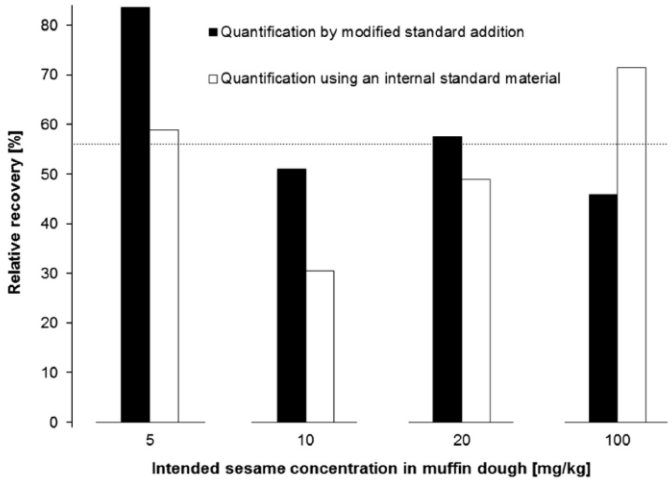

Fig. 4. Relative recoveries of the sesame contents before and after the baking process (adjusted sesame concentrations of 5, 10, 20 and 100 mg/kg in muffin dough). The dotted line represents the mean relative recovery (56%) over all quantification methods and concentration levels.

The limit of quantification is defined as the analyte concentration with a relative uncertainty of the measurement for a confidence interval ($P = 95\%$) $\leqslant 30\%$ (Waiblinger et al., 2001). According to this definition, the LOQ were determined to be 2.4 mg/kg for the matrix-adapted quantification, 6.4 mg/kg for the quantification using an internal standard, and 1.95 mg/kg for the quantification with the modified standard addition.

3.3. Analysis of model foods

Self-prepared muffin doughs with adjusted sesame concentrations of 100, 20, 10 and 5 mg/kg were analysed. The muffin dough material was extracted threefold and analysed in triplicate with each of the quantification methods. Additionally, the muffin dough material was analysed by means of a commercial ELISA kit, to verify the adjusted sesame concentrations in the muffin dough materials. The results are shown in Fig. 3.

The quantification by the modified standard addition provides results in the expected range (recoveries from 59% to 100%) for the dough material. Using the matrix-adapted quantification method, the sesame content in the dough material was underestimated (recoveries from 6% to 11%) for all concentration levels of the muffin dough. These results confirm the recoveries observed for the boiled sausage material. The quantification using an internal standard also shows results in the expected range (recoveries from 85% to 129%), but with high standard deviations ranging from 39% to 71%. The results for the quantification using the commercial ELISA kit confirm the adjusted sesame concentrations in the dough material for 5, 10 and 100 mg/kg (recoveries of 86%, 86% and 92%, respectively). Only for the material with an adjusted sesame concentration of 20 mg/kg, a high recovery of 152% is observed.

One half of the dough material was baked and the muffins were analysed as described above for the dough material. The muffin dough and the muffins were weighed before and after the baking process and the weight differences ranging from 13% to 21% were attributed to a loss of water in the samples. The weight used for the DNA extraction was maintained and thus the increased sesame concentration in the muffins not considered. The increase of the

sesame concentration was determined by the water loss. In order to compare the results for dough and muffins, the quantification results of the muffins were corrected to the corresponding sesame concentrations in the dough. By dividing the corrected sesame concentrations in the muffins by the respective sesame concentrations in the muffin dough, the relative recoveries after the baking process were calculated. The results are depicted in Fig. 4. For the matrix-adapted quantification, the water loss could not be taken into account due to the fact that the sample material was not exactly weighed. The comparability of the sample and the matrix material was assured through the normalization of the concentration of the DNA extracts. Only DNA-containing material effects the DNA normalization, so the water loss could not be considered using the matrix-adapted quantification. The means of the relative recoveries over the 4 concentration levels are 60% for the quantification using the modified standard addition and 52% for the quantification using the internal standard material. The overall mean value for the relative recoveries of the applied quantification methods is 56%. Assuming an equal recovery rate in muffin dough and the corresponding muffins, the difference of −44% of the sesame contents after the baking process might be caused by a heat-induced degradation of the DNA.

4. Conclusion

To the authors' knowledge, a comparative assessment of DNA-based quantification methods in the field of food allergen analysis under pre-defined experimental framework conditions was performed for the first time. Three quantification methods, the matrix-adapted quantification, the quantification using an internal standard material and the quantification by modified standard addition were assessed using uniform performance criteria.

The matrix-adapted method shows a systematic divergence, when the model-matrix differs from the sample matrix. The trueness of the quantification results depends on the similarity of the matrices. In principle, the corresponding model-matrix for each examined sample or at least for each type of food (for example boiled sausages) would be needed. This is reflected in the extreme underestimation of the sesame content in boiled sausages and muffin dough using breadcrumbs as model-matrix.

In contrast, the quantification method using an internal standard material does not depend on a model-matrix. However, the coefficient value needs to be determined for each batch of internal standard material. In addition, high standard deviations of the determined sesame contents were observed. The quantitative results are calculated from initial target sequence copy numbers determined using two independent real-time PCR systems. Therefore, the variability of the copy numbers of both real-time PCR systems contributes to the variation of the quantitative results.

Only the analyte of interest is needed for the quantification using the modified standard addition, therefore, the quantitative results do not depend on a model-matrix. Recoveries in the recommended range of 70–120% were observed during the validation of the method. The addition of the analyte to individual aliquots of the sample material requires, however, at least four PCR analyses per sample. Since only a DNA sequence specific for the analyte needs to be detected, existing real-time PCR methods can be used for the quantification of the allergen content without modifications.

In conclusion, the quantification by modified standard addition emerged from the comparative assessment of the different quantification approaches as the most appropriate method for the determination of the sesame content in food. A general extension of this conclusion to other analytes would require additional validation experiments. However, the data indicate that the quantification via modified standard addition may constitute a basis for the surveillance of the compliance with potential future thresholds for allergenic foods.

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4.2 Publication II

Florian Luber, Anja Demmel, Katrin Pankofer, Ulrich Busch, Karl-Heinz Engel

Simultaneous quantification of the food allergens soy bean, celery, white mustard and brown mustard via combination of tetraplex real-time PCR and standard addition.

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Summary

Food allergies are a worldwide health concern. To protect the consumer, potential allergenic foods have to be labeled by legal administration of governments all around the world. To be able to distinguish unintentional cross-contaminations from intentional usage as ingredient, applicable thresholds are required. For the surveillance of such thresholds appropriate analytical methods are needed. As the standard addition method emerged as the most appropriate quantification approach from previous studies, a tetraplex real-time PCR system was established. To overcome the method's disadvantage of being very laborious, the quantification of soy bean, celery, white mustard and brown mustard was performed simultaneously. The method was validated in spiked boiled sausage material in the range from 40 to 400 mg/kg, and the performance criteria recovery, repeatability, robustness and sensitivity were determined. The evaluation of the validation data proved the method to generate precise data down to the limits of quantification in the range of 2 to 40 mg/kg. The method's suitability as routine approach was proved by qualitative screening and of subsequent analysis of the detected food allergens in commercial food samples.

Candidate's contribution

Evaluation and selection of real-time PCR methods for detection of celery, soy bean, white mustard and brown mustard; establishment of a tetraplex real-time PCR for the simultaneous detection; screening and quantification of commercially available foods; evaluation of the results and statistical assessment; interpretation of the data set;

drawing up and compilation of Figures and Tables; writing of the manuscript; revision of the submitted manuscript.

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Simultaneous quantification of the food allergens soy bean, celery, white mustard and brown mustard via combination of tetraplex real-time PCR and standard addition

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ABSTRACT

Food allergies are a significant public health concern throughout the world with various symptoms that range from mild to severe or even life-threatening. As the only option for the allergenic consumer is the strict avoidance of the allergenic food, in the European Union the declaration of the most frequent food allergens on the labels of products is required. However, traces of food allergens that may be present in foods due to cross-contact are not included in this regulatory provision. Therefore, thresholds above which labeling is mandatory are being discussed. The surveillance of such thresholds requires specific, sensitive and quantitative methods. For this purpose, a tetraplex real-time PCR method was established to quantify simultaneously trace amounts of the four allergens soy bean, celery, white and brown mustard. The quantification is achieved by using the standard addition method. The approach was validated with DNA extracted from lysate mixtures of boiled sausage and the standard materials at concentration levels of 1, 5, 10, 20, 40, 100 and 400 mg/kg. The parameters recovery, repeatability and robustness were evaluated and the limits of quantification of soy bean (8.5 mg/kg), brown mustard (2.6 mg/kg), celery (3.7 mg/kg) and white mustard (36.8 mg/kg) were determined. The method was applied to commercial food products labeled with one or more of the analytes or with a "may contain traces of …" statement.

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1. Introduction

Food allergy is a significant public health concern throughout the world affecting about 3–6% of the population (Sicherer, 2011). Various symptoms can be elicited by a food allergy; they may range from mild to severe or even be life-threatening (Boye, Danquah, Thang, & Zhao, 2012). A cure of food allergies is not yet possible, so the strict avoidance of the allergenic food is the only option for patients (Burks et al., 2012). In order to protect the consumers from an unintentional consumption, in the European Union (EU) the most common allergenic ingredients need to be labeled according to the Directive 1169/2011 (European Parliament and Council of the European Union, 2011). In this Directive, 14 foods and food groups are listed for which labeling is mandatory; the list includes celery, mustard and soy bean. However, this regulatory provision does not consider traces of allergens which entered the product unintentionally through cross-contact. Due to their duty of care, manufacturers often label their products with the term "may contain". The excessive usage of such precautionary labeling leads to uncertainty amongst consumers. Consequently, thresholds concerning allergens arising from cross-contacts are being discussed (Richter, Rubin, & Lampen, 2012), as the implementation of thresholds above which labeling is mandatory might help to reduce the uncertainty of consumers and to facilitate the assessment of the product safety by manufacturers. However, for the surveillance of potential future thresholds, specific and quantitative methods are necessary.

In routine analysis of allergens in foods, enzyme-linked immune sorbent assays (ELISA) and methods based on polymerase chain reactions (PCR) play the most important roles (Fuchs, Cichna-

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Markl, & Hochegger, 2010). Several PCR methods have already been published for the detection of celery (Fuchs, Cichna-Markl, & Hochegger, 2012; Hupfer, Waiblinger, & Busch, 2007; Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008), soy bean (Gryson, Messens, & Dewettinck, 2008; Köppel et al., 2010), white mustard (Fuchs et al., 2010) and brown mustard (Palle-Reisch, Wolny, Cichna-Markl, & Hochegger, 2013). Kits allowing for the quantification of soy bean and celery (CONGEN, 2013a; CONGEN, 2013b) and the detection of mustard (CONGEN, 2013c) are commercially available. For the detection and quantification of soy bean and white mustard, respectively, singleplex real-time PCR systems based on an external calibration have been published (Federal Office of Consumer Protection and Food Safety (BVL), 2013). The disadvantage arising from the fact that this approach is matrix-dependent has been compensated for by using the same sample and calibration materials. In routine analysis, however, this might be difficult to achieve, owing to the high diversity of potential food matrices.

Köppel et al. (2010) established a tetraplex real-time PCR system to simultaneously detect and quantify DNA from celery and soy bean besides hazelnut and peanut. The same authors extended this tetraplex real-time PCR system to a hexaplex system for the detection and quantification of DNA from soy bean, celery, white mustard, cashew, peanut and hazelnut. However, the included mustard system shows some cross-reactivity with other members of the *Brassicaceae* family. In addition, the method does not allow to quantify the allergen concentration in food, because the content of the allergenic food cannot be extrapolated from the DNA amount (Köppel, Velsen-Zimmerli, & Bucher, 2012).

Besides the quantification with an external calibration, two further DNA-based quantification approaches have been developed in order to calculate quantitative data from the cycle threshold values and to overcome the dependency on appropriate standard materials corresponding to the respective food matrices. One of the quantification methods is based on the addition of a unique internal standard material (Hirao, Hiramoto, Imai, & Kato, 2006), the other one on the principle of standard addition (Eugster, 2010).

The aim of this study was the establishment of a tetraplex real-time PCR method allowing the simultaneous quantification of trace amounts of celery, soy bean, white and brown mustard. The method should be based on a modified standard addition procedure in order to be independent from an external calibration. Method development was performed using boiled sausages as matrix; the suitability of the method was demonstrated by investigation of commercially available foods containing celery, mustard or soy bean as ingredients or being labeled with the term "may contain traces of celery, mustard and soy bean".

2. Material and methods

2.1. Material

2.1.1. Sample material

Boiled pork sausages (boiling temperature: 100 °C; boiling time 1.5 h) of the type Lyoner (French: cervelas) were provided by the CVUA Freiburg (Chemisches- und Veterinäruntersuchungsamt, Freiburg, Germany). White mustard seeds, brown mustard seeds, celery seeds and soy flour were obtained from local supermarkets. Commercially available samples named "mixed spices for fried noodles Thai style", "fried noodles with chicken", "mashed potatoes with meatballs" and "noodles Asian style" were purchased in a local supermarket. They were either labeled to contain one or more of the analytes as ingredients or with a "may contain traces of …" statement ("may contain traces of celery, soy bean and mustard"/ "may contain traces of mustard"). The sample materials were ground with a hand blender (M160, ESGE, Metten, Switzerland) and stored at −20 °C until use.

2.1.2. Oligonucleotides

The employed primers and probes are listed in Table 1. To enable a simultaneous detection, specific fluorescence labels were used: The probe of the celery-specific system was labeled with FAM, the probe of the brown mustard system was labeled with ROX, and a HEX-labeled probe was used for white mustard. BBQ was used as a quencher. The probe of the soy bean specific real-time PCR was labeled with ATTO 425 and quenched with DDQI. The lectin probe was obtained by biomers.net GmbH (Ulm, Germany), the other primers and probes were purchased from TIB MOLBIOL GmbH (Berlin, Germany).

2.2. Methods

2.2.1. DNA extraction and standard addition

4.00 g of homogenized boiled sausage was weighed into a 50 mL Falcon-tube. 20 mL CTAB buffer [20 g/L cetyltrimethylammoniumbromide (CTAB), 1.4 mol/L sodium chloride, 0.1 mol/L tris(hydroxymethyl)-aminomethane (TRIS) (Trizma base), 0.02 mol/L disodium ethylenediaminetetraacetate (Na_2-EDTA) (99+%); pH 8.0] and 60 μL proteinase K (recombinant, Roche Diagnostics GmbH, Mannheim, Germany) was added and the mixture was incubated over night at 65 °C under shaking. In parallel, 4.00 g of the analytes (celery seeds, soy flour, white and brown mustard seeds) were treated in the same way. After centrifugation at 7000 g for 10 min, the lysate mixtures of the boiled sausages and the analytes were prepared. The supernatants of the analytes were

Table 1
Oligonucleotides for the detection of celery, soy bean, white and brown mustard.

System	Function/Name	DNA-sequence	Length	Concentration in the reaction mix	Reference
White mustard	Forward primer/MADS D F	5′-TGAAAACTCTCTTCCCCTCTTAGG-3′	24 bp	0.100 μM	(Fuchs et al., 2010)
	Reverse primer/MADS D R	5′-ACAAATGCACACAAGACAGAGATATAGA-3′	28 bp	0.100 μM	
	Probe/MADS D pr	5′-HEX-TACATGATGCTTACCTCGC-BBQ-3′	19 bp	0.120 μM	
Brown mustard	Forward primer/11f	5′-GTTGAGCCGAGGGTCATAATTTC-3′	23 bp	0.300 μM	(Palle-Reisch et al., 2013)
	Reverse primer/11r	5′-TCGACTTAGGCATCCTTACGG-3′	21 bp	0.300 μM	
	Probe/11pr	5′-ROX-CGAGAGTCCGAATACTGGGCTGGGGTC-BBQ-3′	27 bp	0.050 μM	
Celery	Forward primer/Cel-MDH-iF	5′-CGATGAGCGTGTACTGAGTC-3′	20 bp	0.300 μM	(Hupfer et al., 2007)
	Reverse primer/Cel-MDH-iR	5′-AATAGGAACTAACATTAATCATACCAAAC-3′	29 bp	0.300 μM	
	Probe/Cel-MDH-probe	5′-6FAM-AACAGATAACGCTGACTCATCACACCG-BBQ-3′	27 bp	0.200 μM	
Soy bean	Forward primer/Lectin-F	5′-TCCACCCCCATCCACATTT-3′	19 bp	0.900 μM	(BVL L08.00-59, 2013)
	Reverse primer/Lectin-R	5′-GGCATAGAAGGTGAAGTTGAAGGA-3′	24 bp	0.900 μM	
	Probe/Lectin probe	5′-ATTO425-AACCGGTAGCGTTGCCAGCTTCG-DDQI-3′	23 bp	0.200 μM	

mixed in an equal ratio and diluted with 1xTE buffer (1x Tris EDTA buffer, AppliChem GmbH, Darmstadt, Germany) to obtain lysate mixtures of the analytes with 1, 0.1, 0.01 and 0.001%. Those mixtures were used for the standard addition as well as for the preparation of spiked lysates with concentrations of 1, 5, 10, 20, 40, 100 and 400 mg of each analyte per kg sausage. The materials were spiked by pipetting after lysis, to overcome the inaccuracy of spiking trace amounts by weighing.

The standard addition was performed by adding 40 μL of the diluted lysate mixtures (1%, 0.1%, 0.01% and 0.001%) of the analytes to four aliquots (400 μL each) of the spiked lysates for the validation or to four aliquots (400 μL each) of the sample lysates for the analysis of the commercial foods. The standard addition of the diluted lysate mixtures results in aliquots 1.1, 1.2, 1.3 and 1.4 per sample material with final analyte concentrations of $x + 0.0909$, $x + 0.00909$, $x + 0.000909$ and $x + 0.0000909$%, whereby x is the initial analyte concentration of the sample material or the spiked lysate, respectively.

400 μL ReadyRed chloroform/isoamylalcohol (MP Biomedicals Europe, Illkirch, France) was added to the mixtures. After mixing for 30 s and subsequent centrifugation at 17,000 g for 10 min, 375 μL of the clear supernatant was added to 400 μL cold isopropanol (≥99%, SIGMA-ALDRICH GmbH, Steinheim, Germany); the mixture was gently shaken and incubated for 30 min at 7 °C. After centrifugation at 17000 g for 10 min, the isopropanol was discarded and 500 μL ethanol (70%) was added to the residual pellet. The sample was shaken in an overhead shaker and centrifuged again at 17000 g for 5 min. After removing the ethanol, the pellet was dried in a vacuum centrifuge (Eppendorf, Hamburg, Germany) for 10 min (setting: V−Al, 30 °C) and dissolved in 100 μL 1xTE buffer. To reveal potential cross-contamination during the extraction process, an extraction blank, that is without sample material, but with all reagents, was co-extracted for each extraction series.

2.2.2. DNA purification and normalization

The dissolved DNA was purified using the QiaQuick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the DNA was eluted in 25 μL elution buffer.

After the purification of the DNA extracts, the DNA concentration was measured using the fluorometrical intercalating dye Quant-iT PicoGreen dsDNA reagent (Invitrogen, Karlsruhe, Germany). λ-DNA (Invitrogen, Karlsruhe, Germany) was used for the generation of a standard curve. The fluorescence was measured by a GENious plus reader (Tecan, Männedorf, Switzerland) at a wavelength of 520 nm. The calculation of the DNA concentration of the samples was performed using the resulting linear equation of the standard curve. To overcome differences in the extraction yields between individual extractions, the DNA extracts were normalized with 1xTE buffer to a concentration of 20 ng/μL and stored at −20 °C until use.

2.2.3. Tetraplex real-time PCR

The reaction mixture consisted of 20 μL mastermix and 5 μL DNA template. The concentrations of the oligonucleotides were adopted from the original publications, except for white mustard, for which the concentrations from the original publication were reduced to obtain an efficiency in a range similar to the other real-time PCR systems combined in the tetraplex approach (Table 1). Each reaction was carried out in a well of a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems, Foster City, USA). All extracts were tested in duplicate. Additionally, every plate contained six standards in duplicate, an extraction blank value and a no template control (NTC). After sealing with MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, USA), the plate was centrifuged for 1 min at 100 rcf (Universal 320. Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). The real-time PCR was performed using a Stratagene MX3005P cycler (Agilent Technologies, Waldbronn, Germany). The temperature program was as follows: Polymerase activation at 95 °C for 15 min, followed by 45 cycles with 94 °C for 30 s and 60 °C for 60 s. After each cycle, the fluorescence intensities were measured.

The software MxPro − Mx3005P v4.10 (Applied Biosystems, Foster City, USA) was used for data analysis. The thresholds and Ct values were determined automatically by the software using the settings “adaptive baseline” and “amplification based threshold”.

2.2.4. Validation

The quantification via combination of tetraplex real-time PCR and standard addition was validated with lysate mixtures of boiled sausage and standard materials (celery, soy bean, white and brown mustard). After the standard addition, the DNA was extracted from each combination of lysate mixtures and analyzed by means of real-time PCR. In every run, a standard curve was obtained by analyzing six serial dilutions of a mixture containing DNA from all of the standard materials. The efficiency calculated from the slope of the standard curve was used in the calculation of the allergen contents.

2.2.4.1. Recovery. Two extractions were carried out for the concentration levels of the lysate mixtures of 400, 100 and 40 mg/kg, respectively. In the real-time PCR, for each concentration level two extractions were performed and eleven replicates were analyzed. The experimentally determined analyte concentrations in the lysate mixtures were compared to the adjusted concentrations.

2.2.4.2. Repeatability. The DNA of lysate mixtures of boiled sausages mixed with lysate of standard material with concentrations of 400, 100 and 40 mg/kg was extracted twice. On the first day, each level of standard addition of each extract was measured five times by means of real-time PCR. On the second day, the real-time PCR analysis was repeated with three replicates (ENGL, 2011). For each analyte concentration, the RSD_r was calculated from the quotient of the determined standard deviations and the means, weighted by the sample size (Luber et al., 2014).

2.2.4.3. Robustness. Two extractions were carried out for the concentration levels of the lysate mixtures of 400, 100 and 40 mg/kg, respectively. In the real-time PCR, five replicates were measured for each level of standard addition and extraction. A second operator pipetted three further replicates for the analysis by means of real-time PCR. The means of the concentrations determined by the first and the second operator were calculated. The quotient of the difference of these means and the mean of all values determined by both operators was calculated and results in the relative deviation of the means.

2.2.4.4. Limit of quantification. Two extractions were carried out for the concentration levels of the lysate mixtures of 20, 10, 5 and 1 mg/kg, respectively. In the real-time PCR, five replicates were measured for each level of standard addition and extraction. For each concentration level and analyte, the deviation of the values for a confidence interval of 95% was determined and plotted against the respective concentration. The LOQ is then the intersection point of the curve with the straight line of $y = 30\%$. The equation of the curve was used for the calculation of the LOQ by inserting 0.3 into the equation as value y (Waiblinger, Gutmann, Hädrich, & Pietsch, 2001).

3. Results and discussion

3.1. Principle of the quantification approach by tetraplex real-time PCR

The tetraplex real-time PCR is composed of four real-time PCR systems for the detection of celery, soy bean, white and brown mustard. The system for white mustard is based on the mRNA gene for the MADS D protein (Fuchs et al., 2010). The brown mustard system detects the *Brassica nigra* partial RT gene for reverse transcriptase from gypsy-like retroelement 13G42-26 (Palle-Reisch et al., 2013). The celery system is based on the mannitol dehydrogenase gene of celery (Hupfer et al., 2007). The soy bean system is based on the lectin gene of soy bean (Federal Office of Consumer Protection and Food Safety (BVL), 2013). This tetraplex real-time PCR setup was combined with a standard addition method to achieve the simultaneous quantification of the four allergenic foods. The standard addition as described by Eugster (2010) was modified as follows: (i) The DNA was extracted with a CTAB-based extraction method and purified with the QIAquick purification kit. (ii) The concentrations of the added lysates of the standard material to the four sample aliquots were modified to match the lower concentration range of interest from 10 to 10,000 mg/kg (iii) In contrast to the published method, where an efficiency of 90% was assumed (Eugster, 2010), the efficiency was determined for each analyte in each analysis series and used in the calculation of the quantitative results.

The functionality of the tetraplex real-time PCR was demonstrated by a series of experiments. At first, a serial dilution (ranging from 100 to 0.01 ng per reaction) of the DNA from brown mustard was analyzed by means of singleplex real-time PCR. In subsequent steps, DNA from white mustard, celery and soy bean was added to the dilution series and the resulting mixtures were analyzed by means of the respective duplex, triplex and tetraplex real-time PCR-systems. As shown in Fig. 1, the sequential addition of further analytes had no negative influence on the efficiencies of the real-time PCR systems for brown mustard, celery and soy bean; only the efficiencies of the white mustard system varied from 85 to 102%, depending on the number of real-time PCR systems combined. The particularly low efficiency of 85% determined for white mustard in the triplex-system may result from the fact that no ct value for the lowest concentration of analyte DNA (0.01 ng/reaction) could be determined under these experimental conditions and that this affected the calculation of the efficiency from the linear equation of the dilution series. Apart from this exception, all efficiencies were in the range of 90–110%, which is required by the ENGL working group (ENGL, 2008) for quantitative real-time PCR. The described experiments only cover one of the possible orders in the course of the sequential addition from singleplex to tetraplex real-time PCR. Therefore, further experiments would be necessary to reveal the potential influence of the combination of real-time PCR systems and analytes on the slight fluctuations of the calculated efficiencies for each of the four real-time PCR systems used in singleplex, duplex, triplex and tetraplex, respectively. For the tetraplex real-time PCR system, actually used for the simultaneous detection and quantification of the four analytes, the efficiencies for the four analytes ranged from 90.5 to 96.6%. In addition, the calibration curves of the tetraplex-system showed sufficient linearities over the whole dynamic range ($R^2 \geq 0.98$). Therefore, the tetraplex-system was considered suitable for the quantification of the allergenic foods celery, soy bean, white mustard and brown mustard.

3.2. Validation of the quantification method

3.2.1. Recovery

According to the Working Group of the European Network of Genetically Modified Organism Laboratories (ENGL, 2008), the recovery is defined as the compliance of the average value determined by means of a series of test results and an acknowledged reference value. For GMO analysis, it shall be in the range of 75–125% of the accepted reference value over the whole dynamic range. The AOAC Food Allergens Analytical Community considered recoveries between 50 and 150% to be acceptable for quantitative food allergen ELISA methods (Abbott et al., 2010). As no performance criteria for the quantification of food allergens by means of real-time PCR have been established up to the present, both criteria were applied to the validation of the tetraplex real-time PCR.

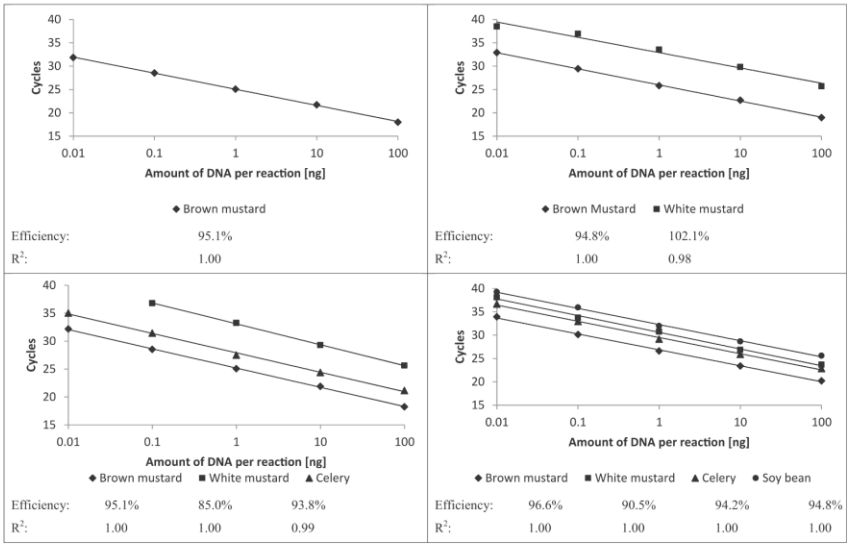

Fig. 1. Calibration curves of brown and white mustard, celery and soy bean by means of singleplex, duplex, triplex and tetraplex real-time PCR, respectively, from dilution series of 100 to 0.01 ng DNA per reaction.

Taking the standard deviations into consideration, the observed recoveries are comparable to the requirements of the AOAC International; even the mean recovery at the concentration level of 400 mg soy bean per kg sample (165%) does not differ significantly ($\alpha = 0.05$) from 150% (Fig. 2). The recoveries obtained in the tetraplex system are in the same order of magnitude as those reported for commercial singleplex quantification kits available for soy bean (77–102%) (CONGEN, 2013a) and celery (82–116%) (CONGEN, 2013b). For 7 of the 12 samples the recovery rates were within the acceptance criteria of the ENGL working group. However, it has to be kept in mind that these acceptance criteria are inferred from the analysis of GMO, where the concentration range of interest (decision level: 0.9%) is higher. For the trace analysis of allergens, these criteria may therefore be used as a starting point of reference, but a strict compliance cannot be required.

3.2.2. Repeatability

The ENGL working group describes the repeatability as the relative standard deviation of test results obtained under repeatability conditions (ENGL, 2008). Repeatability conditions are summarized as conditions where test results are achieved with the same method, on identical test items, in the same laboratory, by the same operator, with the use of the same equipment within short intervals of time. The relative repeatability standard deviation (RSD_r) shall be ≤25%.

For soy bean the RSD_r of the tetraplex real-time PCR determined for the concentration levels 40–400 mg/kg analyte in sausage lysate ranged from 11.9 to 17.7% (Fig. 3). They are in the same order of magnitude as those reported for the available commercial singleplex quantification kit (12.1–22.1%, for 4–400 mg/kg analyte in cornmeal) (CONGEN, 2013a). The repeatability of the tetraplex real-time PCR for celery was determined to be 10.5% for 40 mg/kg analyte in sausage and 18.1% for 400 mg/kg. In comparison, the validation reports of the commercial quantification kit state 15% for 40 mg/kg celery in cornmeal and 14% for 400 mg/kg (CONGEN, 2013b). In general, the RSD_r of the white mustard system were significantly higher than those for the other analytes. For the concentration levels of 40 and 100 mg white mustard per kg sample the RSD_r exceeded the threshold of 25% established by the ENGL working group. The RSD_r of all three concentration levels of brown mustard were the lowest. A comparison with other methods is not possible, as no data of quantitative real-time PCR methods for brown mustard are available so far.

3.2.3. Robustness

The robustness of a method is expressed by its capacity to remain unaffected by small deviations from the applied experimental conditions, e.g. different thermal cycler model or operator.

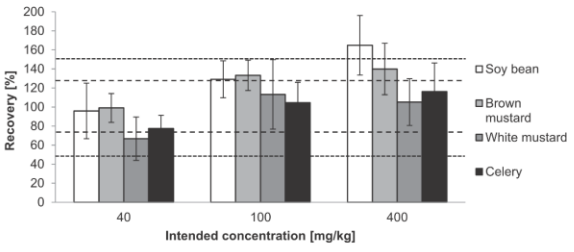

Fig. 2. Recoveries of soy bean, celery, brown and white mustard spiked in concentrations of 40, 100 and 400 mg/kg to lysate mixtures of boiled sausage (dotted lines: Acceptance criteria of the AOAC International; dashed lines: Acceptance criteria of the ENGL working group).

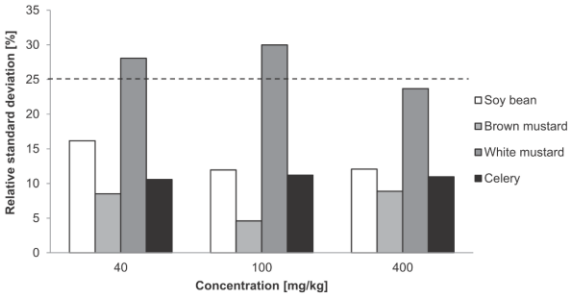

Fig. 3. Relative standard deviations under repeatability conditions of the determined concentrations of soy bean, celery, brown and white mustard in lysate mixtures of boiled sausages (dashed line: Acceptance criteria of the ENGL working group).

According to the European Network of Genetically Modified Organism Laboratories Working Group, the obtained deviation from the test result shall be within ±30% (ENGL, 2008).

The relative deviations of the means of the different analyte concentrations are depicted in Fig. 4 and ranged from 3% (soy bean 100 mg/kg) to 29% (white mustard 100 mg/kg). The threshold value of 30% established by the ENGL working group could be fulfilled for every analyte concentration.

3.2.4. Limit of quantification

The limit of quantification (LOQ) corresponds to the lowest concentration or amount of analyte in a sample, its quantification being reliable with an acceptable level of accuracy and precision (ENGL, 2008). The LOQ has been defined as the concentration for which the deviation of the values is below 30% for a confidence interval (CI) of 95% (Waiblinger et al., 2001).

The LOQ of soy bean was determined to be 8.5 mg/kg and the resulting LOQ of brown mustard was 2.6 mg/kg. The determination of the LOQ of white mustard yielded a concentration of 36.8 mg/kg, whereas the LOQ of celery was 3.7 mg/kg. As for the commercial singleplex real-time PCR systems, the sensitivities for the tetraplex real-time PCR system were in the low mg/kg range as relevant to the detection of food allergens (CONGEN, 2013a; CONGEN, 2013b; Poms, Klein, & Anklam, 2004). The LOQs of the commercial real-time PCR systems for the detection of celery and soy bean were determined theoretically from standard curves of dilution series with a known concentration and may therefore vary depending on the matrix (CONGEN, 2013a).

The LOQ of white mustard was approximately tenfold higher than those of the other three analytes. However, the LOQ of white

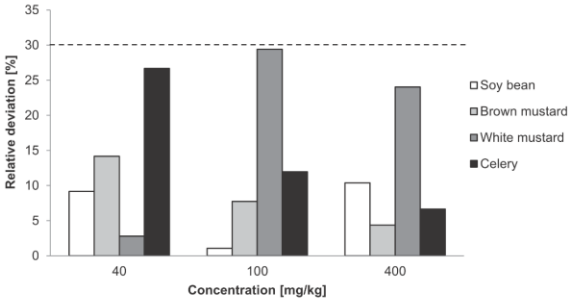

Fig. 4. Relative deviation of the means of the concentrations of soy bean, celery, brown and white mustard for the determination of the robustness of the method (dashed line: Acceptance criteria of the ENGL working group).

mustard is also still in the range of the upper limits of 10–100 mg/kg of the allergenic food or 1–10 mg/kg of the protein fraction, that are considered by the "food allergy" working group of the German Society for Allergology and Clinical Immunology and of the Association of German Allergologists to protect most allergic consumers from severe allergic reactions (Vieths et al., 2006).

The validation data of the tetraplex real-time PCR show that the modified standard addition method by Eugster (2010) is applicable for the quantification of soy bean, celery, white and brown mustard using boiled sausages employed as sample material. In comparison with the commercial quantification kits for soy bean and celery, the validation parameters are within the same range, except for the LOQs that are lower for the commercial quantification kits. However, the quantification by means of these two kits is matrix-dependent. It is based on allergen-containing cornmeal as reference material which does not correspond to the matrix of most foods under consideration; therefore, the transferability of the cornmeal-based results would need to be confirmed before the kits can be applied to other matrices.

The recovery rates, the relative repeatability standard deviations, the relative deviations of the means and the limits of quantification show differences between the respective analytes. They may result from the presence of four different real-time PCR systems in one chemical reaction which can lead to mutual influence of the systems. Additionally, the target sequences of the respective real-time PCR systems may be present in different copy numbers in the genome which influences the sensitivity of the real-time PCR. A further reason may be the different efficiency of the amplification associated with the secondary structure of the respective target sequences and the related annealing temperatures. The secondary structure results from the amount of the nucleobases guanine and cytosine and therefore varies for every real-time PCR system (Yakovckuk, Protozananova, & Frank-Kamenetskii, 2006). The differences between the concentration levels result from the low concentration range in which the quantification is performed. At this low concentration, only a few DNA copies per target are present which leads to an increase of the coefficient of variation and, therefore, a higher variability of the values (Horwitz, 1982).

3.3. Screening and quantification of soy bean, celery, white and brown mustard in commercial food samples

The validated tetraplex real-time PCR was applied to the analysis of commercially available samples which were either labeled with one or more of the analytes as ingredients or with a "may contain traces of …" statement ("may contain traces of celery, soy bean and mustard"/"may contain traces of mustard"). The samples were screened for the allergens soy bean, celery, brown and white mustard using qualitative real-time PCR. The detected allergenic ingredients were then quantified using the developed combination of real-time PCR and standard addition. For the qualitative analysis, the sample DNA was extracted once and the tetraplex real-time PCR was performed in triplicates (Table 2). The subsequent quantification by means of standard addition was achieved from three DNA extractions. For each extraction, two replicate series were analyzed by means of tetraplex real-time PCR. The serially diluted DNA standards were also measured in duplicate. The concentrations were calculated separately for each replicate series. As example, Table 3 shows the results for the quantification of celery in a commercially available food sample. Four aliquots of the sample material are spiked with different dilutions of the supernatant of celery resulting from incubation with CTAB buffer and Proteinase K. From the ct values of the four aliquots the amount of celery in the sample is determined by approximation calculation as described by Eugster (2010). This results in 6 values (2 replicates from 3 extractions) out of which the mean and standard deviations are calculated. In advance, the Cochran's C test was applied to test the variance homogeneity of the three extractions depending on each individual analyte (Kromidas, 2000).

The screening results of the sample material "mixed spices for fried noodles Thai style" are shown in Table 2. On this product curry has been labeled as an ingredient, containing celery and mustard, which were confirmed by the qualitative analysis with mean ct (ct_{mean}) values of 30.85 (white mustard), 36.75 (brown mustard) and 32.58 (celery). Although soy beans were labeled as components of powdered soy sauce, soy bean could not be detected. The quantitative analysis resulted in amounts of 4005 ± 2148 mg/kg white mustard and of 1411 ± 637 mg/kg celery (Table 4). The amounts of 3 ± 1 mg/kg quantified for brown mustard confirmed the assumed traces indicated by the high ct values (ct_{mean} = 36.75) of the qualitative analysis.

The sample "fried noodles with chicken" was screened positive for all four analytes, which is in accordance with the labeling indicating celery, mustard and powdered soy sauce as ingredients. The subsequent quantitative analysis determined the concentration of white mustard to be 1034 ± 57 mg/kg and of celery to be 338 ± 69 mg/kg. No quantification of soy bean was possible due to the fact that no increase of fluorescence for at least two PCRs within one replicate series could be measured and, therefore, the standard addition procedure could not be applied. The amount of brown mustard was below the LOQ of 2.6 mg/kg.

The precautionary labeling "may contain traces of celery, soy bean and mustard" has been used on the product "mashed potatoes with meatballs". The screening of the sample material detected the presence of celery and mustard, but not of soy bean. The content of celery was determined to be 67 ± 6 mg/kg and that of white mustard 136 ± 31 mg/kg.

Table 2
Qualitative analyses of commercially available food samples by means of tetraplex real-time PCR.

Food sample	Qualitative analyses of PCR triplicates for each analyte [ct values]			
	Soy bean	White mustard	Brown mustard	Celery
Mixed spices for fried noodles Thai style	no Ct	30.71	37.21	32.62
	no Ct	30.90	36.87	32.44
	no Ct	30.95	36.18	32.69
Fried noodles with chicken	40.73	30.75	36.96	32.33
	37.99	30.53	38.52	32.11
	37.97	30.69	37.73	31.88
Mashed potatoes	no Ct	31.44	36.69	30.76
with meatballs	no Ct	31.61	38.21	31.05
	no Ct	31.87	36.99	30.64
Noodles Asian style	35.45	38.99	40.85	31.21
	34.56	no Ct	37.88	31.14
	35.01	no Ct	38.53	31.19

Table 3
Ct values for the quantitative analysis of celery in fried noodles with chicken by means of tetraplex real-time PCR and standard addition.

	Ct values				Resulting amount [mg/kg]	Mean ± standard deviation [mg/kg]
	Aliquot 1.1	Aliquot 1.2	Aliquot 1.3	Aliquot 1.4		
Extract 1						338 ± 69
Replicate a	32.66	34.12	35.01	34.84	261	
Replicate b	32.89	33.94	34,44	34.95	415	
Extract 2						
Replicate a	33.10	34.85	34.99	35.51	263	
Replicate b	33.22	34.58	34.82	35.27	373	
Extract 3						
Replicate a	33.03	34.75	34.87	35.04	313	
Replicate b	33.06	35.34	34.43	34.45	405	
No Template Control:	No Ct	**Parameters of the standard curve:** Efficiency = 98.40% R^2 = 0.997				
Extraction Blank:	No Ct					

Soy sauce and celery were listed as ingredients of the sample material “noodles Asian style”. In addition, the labeling “may contain traces of mustard” was used. The qualitative analysis of the sample material led to positive results for the presence of the labeled ingredients soy bean and celery. The precautionary labeling of mustard could be confirmed for brown mustard (ct_{mean} = 39.09), but not for white mustard (only one of three results is positive) by qualitative analysis. The following quantification resulted in 134 ± 19 mg/kg for soy bean and 616 ± 219 mg/kg for celery. The content of brown mustard in the sample material was below the LOQ.

Celery has been listed on three of the analyzed commercial products as ingredient and on one product as trace amount. It was detected and quantified in all four sample materials, whereby the lowest amount was determined in the product with the precautionary labeling “may contain traces of celery, soy bean and mustard”.

Two of the investigated commercial samples contain mustard as ingredients, which could be confirmed by qualitative and quantitative analysis of white mustard. Assumed traces due to precautionary labeling could be qualitatively and quantitatively verified for the analysis of white mustard in one of two samples. The presence of brown mustard was determined for all sample materials by qualitative analysis. In contrast to the positive screening results, no quantification of brown mustard was achieved except for “mixed spices for fried noodles Thai style” with a low concentration of 3 mg/kg. The positive screening results of brown mustard may be explained by the high sensitivity of the real-time PCR system that shows the lowest LOQ of all systems. Nevertheless, the results of the qualitative analysis are in good accordance with those of the quantification: The sample “mixed spices for fried noodles Thai style” results in a mean ct value of 36.75 and can be quantified to be 3 mg/kg which is above the method's LOQ of 2.6 mg/kg. All other samples resulted in mean ct values above 36.75 for the qualitative analysis that indicate the presence of brown mustard in very low concentrations which appear to be below the LOQ of the employed tetraplex approach.

Although soy bean was labeled on all products as ingredient or at least as trace amount (“mashed potatoes with meatballs”) the amount of soy bean could be quantified only for one sample material (“noodles Asian style”). This might be due to the fact that powdered soy sauce, a highly processed ingredient, has been used. As a result of the production process that includes a fermentation step of the soy beans and a subsequent spray drying, the DNA might be degraded and thus no detection by real-time PCR might be possible.

4. Conclusion

The results of the commercially available samples demonstrate that the developed tetraplex real-time PCR system in combination with standard addition method is applicable for the quantitative

Table 4
Quantitative analysis of soy bean, celery, white and brown mustard in commercially available food samples by means of tetraplex real-time PCR and standard addition.

Food sample	Labeling	Analyzed analyte	Mean (n = 6) [mg/kg]	Standard deviation [mg/kg]
Mixed spices for fried noodles Thai style	Ingredients: curry (containing celery and mustard) and powdered soy sauce (soy beans, wheat)	Soy bean	–[a]	–[a]
		White mustard	4005	2148
		Brown mustard	3	1
		Celery	1411	637
Fried noodles with chicken	Ingredients: spices (containing celery and mustard) and powdered soy sauce (soy beans, wheat)	Soy bean	–[c]	–[c]
		White mustard	1034[b]	57[b]
		Brown mustard	<LOQ	<LOQ
		Celery	338	69
Mashed potatoes with meatballs	May contain traces of celery, soy bean and mustard	Soy bean	–[a]	–[a]
		White mustard	136[b]	31[b]
		Brown mustard	<LOQ	<LOQ
		Celery	67[b]	5[b]
Noodles Asian style	Ingredients: powdered soy sauce [soy sauce (water, soy beans, wheat, salt)] and celery. May contain traces of mustard	Soy bean	134[b]	19[b]
		White mustard	–[a or c]	–[a or c]
		Brown mustard	<LOQ	<LOQ
		Celery	616	219

[a] Not quantified due to negative screening results.
[b] n = 4; one DNA extract (n = 2) was eliminated as an outlier according to Cochran's test (P = 95%).
[c] Not quantified due no increase in fluorescence for at least two PCRs within one replicate series.

analysis of traces of allergens in foods. The range of efficiencies determined for the four analytes in the tetraplex-system and the elaborated validation data demonstrate the suitability of the method for practical purposes. The variability in recovery rates as observed for soy bean can be taken into account depending on the concentration range determined for the analyte. The method should be a valuable tool for food manufacturers and food safety authorities. The advantage of a multiplex real-time PCR system is the simultaneous analysis of several analytes. Compared to singleplex real-time PCR systems it is time-saving which is important for routine analysis of allergens. In addition, less DNA extract is being used when several analytes are quantified simultaneously. This is advantageous if only small amounts of sample material are available.

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5 Discussion

5.1 Comparative assessment of DNA-based quantification approaches

5.1.1 Matrix-adapted quantification

If the model matrix differs from the matrix of the analyzed sample, a systematic divergence is observed for matrix-adapted quantifications. This was particularly reflected by the performance criterion recovery, for which an underestimation was observed when wheat-based breadcrumbs were used as model matrix for determinations in boiled sausages, muffin dough and muffins (Publication I). Nevertheless, the precision of this method expressed by the repeatability and the reproducibility was shown to be in an acceptable range.

This systematic divergence has also been reported by Siegel et al. (2012) analyzing sauce powder, sausage, rice cookie, wheat cookie and a commercial kit standard. For the quantification, an external matrix standard on the basis of rice cookie was used, resulting in a recovery of 107 ± 6% in rice cookie spiked with 100 ppm sesame. When the examined matrix differed from the applied standard material, recoveries varied from 66 ± 7% for the sauce powder to 127 ± 17% for the commercial kit standard (Siegel et al., 2012).

To overcome the disadvantage of the reported underestimation, the corresponding model-matrix for each examined matrix or at least for each type of food should be available, or conversion factors must be applied. In commercially available real-time PCR kits which are based on this matrix-adapted quantification approach, the quantification is achieved by a matrix-matched calibrator containing 40 ppm of each allergenic food (R-Biopharm, 2019b) that can be co-analyzed by the available real-time PCR kits (R-Biopharm, 2018a, 2018b, 2018c, 2018d, and 2019a). In addition, a multiplex real-time PCR kit is available for the simultaneous detection of soy bean, celery and mustard (R-Biopharm, 2018a). The limit of quantification of the commercial approaches is stated to be 0.4 ppm allergenic food determined in diluted DNA extracts from spiked corn flour. No factors to convert from corn flour-based material to the analyzed food samples are provided.

5.1.2 Quantification using internal standard material

The quantification method using an internal standard material does not require a model-matrix; however the coefficient-value, i.e. the ratio of initial copy numbers of the internal standard material and the target analyte in the DNA extract, needs to be determined. An overestimation of the results for sesame in boiled sausages material was observed, and the precision criteria repeatability ($RSD_r \leq 25\%$) and reproducibility ($RSD_R \leq 35\%$) were only fulfilled for the spiking levels of 20 and 100 mg/kg (Publication I).

The principle of the method is based on the determination of initial copy numbers by two different real-time PCR systems (e.g. for sesame and the internal standard material *Gypsophila elegans*). Therefore, the variability of the two real-time PCR systems used for the calculation of the respective copy numbers contributes to the variation of the quantitative results. The coefficients of variance were determined to be between 24 and 40%, when analyzing 100 ppm of buckwheat in different matrices (Hirao et al., 2006). These data were calculated from three PCR replicates regarding only the deviations of the real-time PCR systems and not addressing the influences of weighing in the standard materials and the subsequent DNA extraction.

Demmel et al. (2012) transferred the principle of quantification developed by Hirao et al. (2006) to the quantification of lupine, an allergenic food with relevance in Europe. Determining the content of lupine flour in wheat flour in the range of 1 to 10 ppm resulted in recoveries of 146 to 186%. As for the quantification of sesame in boiled sausages, also an overestimation could be observed. The relative standard deviation calculated from at least 8 DNA replicate extracts was in the range of 35.3 to 49.3%, with an overall relative standard deviation for all concentrations of 42% (Demmel et al., 2012). This is in good agreement with the mean standard deviation of 36 % for the repeatability determined at the investigated concentration levels of sesame in boiled sausage material (Publication I).

5.1.3 Quantification by competitive real-time PCR

In the meantime, newer approaches have been reported that reflect the progress in the field of quantification via competitive real-time PCR. The comparative PCR method has been improved from a semi-quantitative (Holzhauser et al., 2009) to a quantitative

approach (Holzhauser et al., 2014). The competitor DNA was adjusted to a level that equals 100 mg/kg peanut in chocolate with a quantification range from 10 to 1000 mg/kg. The recovery and the precision data showed valid results for the analysis of sample material containing 100 mg/kg. For concentrations differing from the adjusted calibrator concentration (e.g. concentrations of 10 or 1000 mg/kg), the recoveries and the precision data led to higher deviations (Holzhauser et al., 2014). This quantification method was extended to the quantification of peanut and soy bean in spiked foods (Ladenburger et al., 2018). Mitochondrial DNA was selected as target to improve the method's sensitivity. Competitor molecules were titrated to a copy number that equals 10 ppm of soy bean, respectively peanut, in milk powder. This allowed the quantification of the both allergenic foods in a range of 1 to 100 ppm in spiked sausage, rice cookie, sauce Hollandaise and milk powder. The quantification of soy bean resulted in recoveries ranging from 114 to 290% (mean: 174%) and of peanut from 70 to 360% (mean: 125%). The results elaborated by Holzhauser et al. (2014) demonstrated that the recoveries deviate more if the amount of analyte differs strongly from the value titrated with competitor molecules. Especially for the detection of peanut, excluding the results for the material spiked with 1 ppm and 100 ppm led to recoveries of 78 to 152% (mean: 112%).

5.1.4 Quantification by standard addition

Applying the standard addition method, soy bean was quantified in wheat and rice flour and a mixture of both (spiking level: 0.15, 0.08 and 0.15%, respectively) with recoveries ranging from 77 to 104% and relative standard deviations of 17 to 27%. In addition, the method was tested at a higher concentration of 5.18% soy bean in rice flour resulting in a recovery of 102% (Eugster, 2010). For the quantification of sesame, the method showed recoveries in the range of 72 to 114% (spiking level: 5 to 100 ppm) and precision data that are, at least for spiking levels > 20 ppm, in compliance with the requirements for quantification by means of ELISA methods (Publication I).

Even though the standard addition is very laborious, the precision data and recoveries were very promising. To overcome the main disadvantage of the method, a simultaneous determination by means of tetraplex real-time PCR was established for the quantification of soy bean, celery, white mustard and brown mustard (Publication II). The mean recoveries determined for boiled sausage material spiked

with 40, 100 and 400 mg/kg of each of the analytes were 130% (soy bean), 124% (brown mustard), 95% (white mustard) and 99% (celery). The precision, expressed by the repeatability, i.e. the relative standard deviation under repeatability conditions, was 27.2% (white mustard), 13.4% (soy bean), 7.3% (brown mustard) and 10.9% (celery). In addition, the method's capacity to remain unaffected by small deviations in the applied experimental conditions was proved by the robustness. The mean robustness determined for the spiking levels of 40, 100 and 400 mg/kg was below the value of 30%, which has been defined as performance criterion in the field of GMO analysis (Publication II).

5.2 DNA- versus protein-based methods for analysis of allergens

5.2.1 Reference doses and action values as benchmark for analytical tools

The VITAL program (Taylor et al., 2014) established by the Australian Allergen Bureau is the current basis of allergen risk assessment, providing reference doses of food allergens to protect the vast majority of allergic consumers. These values are derived from an extensive review of available clinical data (Brooke-Taylor et al., 2017). From these data, dose-response curves have been determined applying different statistical modeling approaches (Crevel et al., 2007), allowing for the definition of eliciting doses at which a certain percentage of allergenic people would not react (Brooke-Taylor et al., 2017). This quantitative risk assessment is expressed by the eliciting dose ED01 (no risk for 99% of allergic population) and/or ED05 (no risk for 95% of allergic population) (Taylor et al., 2014; Brooke-Taylor et al., 2017). These values were used as basis for the definition of internal action values for official food control laboratories in Germany (Waiblinger and Schulze, 2018). A conversion factor is applied to convert from total protein of the allergenic food to total allergenic food. Assuming an average consumption amount of 100 g, concentrations of the allergenic food in 100 g serving sizes are determined. Based on these serving sizes the official food control laboratories in Germany have derived the action values expressed as analytical result in mg/kg (Table 2).

Monitoring these thresholds or action values requires appropriate analytical methods that can be divided into protein-based and DNA-based applications. Applied in commercial kits, ELISA methods have become very popular, as they allow the quantification in a practicable way and do not require expensive equipment. Meanwhile, MS-based techniques have been established for the detection and quantification of food allergens. Marker peptides are detected subsequent to the extraction of total protein and tryptic digestion. These marker peptides are matched with allergens, and the content of allergenic protein to total protein can be calculated (Parker et al., 2015).

The detection of egg and milk as sources of potentially allergenic food is not possible with DNA-based methods, since these methods cannot distinguish between DNA from chicken and egg or from beef and milk. Moreover, DNA yields in both, milk and egg,

are rather low. For the detection of gluten as parameter for celiac disease, protein-based ELISA kits are state of the art. The gliadin fraction is firstly extracted and determined and secondly multiplied by two, since gliadin usually accounts for 50% of the proteins in gluten. Nevertheless, ELISA kits cannot differentiate the cereals as source of the gliadin, although labeling in the EU is mandatory for the individual gluten-containing cereals. To close this gap, highly specific multiplex real-time PCR systems are available, which allow for the differentiation of barley, oat, rye, maize, rice, and wheat (Dolch et al., 2019). The specificity of DNA-based approaches refers down to the sequence of nucleotides, i.e. one level below the amino acid sequence as basic constituent of the peptides determined via MS-based techniques or specific epitopes of proteins used as antigens of ELISA methods. For example, the determination of celery, which requires mandatory labeling in the EU, is not possible via ELISA kits, as celery cannot be differentiated from other plants of the *Apiaceae* family (Faeste et al., 2010; Fuchs et al., 2013). Although no MS-based method has been reported so far, a highly specific real-time PCR allows indeed for the detection of celery (Hupfer et al., 2007). Meanwhile, PCR analysis has been established as de facto standard method in Japan and Germany. In Japan, PCR is used as confirmation method of initially applied ELISA kits for buckwheat, crab, peanut, prawn, shrimp and wheat (Sakai et al., 2013). In Germany, PCR is included in the official collection of methods of analysis according to § 64 of the German Food and Feed Act for various allergenic components for which labeling is mandatory.

Table 2: Action values for official food control in Germany based on recommendations of the VITAL Scientific Expert Panel for allergologic assessment (modified from Waiblinger and Schulze (2018))

Allergenic foods	Analytically determined to be [a]	Protein reference dose [mg] [b]	Conversion factor (protein to food) [c]	Converted reference dose food [mg]	Concentration of allergenic food in 100g serving size [mg/kg]	Analytical result [mg/kg] [d]
Cereals containing gluten	Gluten	1.0 (wheat)	10.0	10	100	>80
Eggs and products thereof	Whole egg powder	0.03	2.2	0.066	0.66	>1
Peanut and products thereof	Peanut	0.2	4.0	0.8	8	>5
Soy beans and products thereof	Soy flour	1.0	2.5	2.50	25	>20
Milk and products thereof (including lactose)	Defatted milk powder	0.1	2.8	0.28	2.8	>2.5
Hazelnut	Whole nut	0.1	6.4	0.64	6.4	>5
Cashew	Whole nut	2.0	5.3	10.6	106	>50
Almonds, walnuts, pecan, Brazil nuts, pistachio, and macadamia nuts	Whole nuts	Not specified				>20 [e]
Sesame seeds and products thereof	Whole seeds	0.2	5.9	1.18	11.8	>10
Celery and products thereof	Celery seeds	Not specified				>20 [e]
Mustard and products thereof	Mustard seeds	0.05	3.8	0.19	1.9	>5

[a] Conversions to the specified type of material may be necessary, especially when using commercial ELISA kits (e.g. specified in the kit or available protein reference values for the foodstuff from the literature) (Waiblinger & Schulze, 2018)

[b] Protein reference doses in terms of milligrams of total protein of the allergenic food, derived from statistical dose distribution models based on eliciting doses according to the recommendations of the VITAL Scientific Expert Panel (Taylor, et al., 2014)

[c] Conversion from protein to allergenic food on the basis of published conversion factors (Taylor, et al., 2014)

[d] Quantifiable positive result of an allergenic constituent for which the expert laboratory report provides recommendations for further investigations; action values represent internal values of official food control laboratories in Germany with no demands on legal threshold values (Waiblinger & Schulze, 2018)

[e] Currently no reference dose available; preliminary value only based on analytical feasibility (Waiblinger & Schulze, 2018)

Both analytical techniques, protein-based and DNA-based methods, are required to be able to detect allergenic foods for which labeling is mandatory. In general, DNA-based methods are rather used for qualitative analysis and protein-based methods for quantification purposes. The latter are stated as direct analytical tools as these methods determine the protein itself, whereas DNA-based approaches detect a species-specific sequence of the DNA as indicator for the allergenic food. However, it has to be kept in mind that different conversion factors have to be applied when analyzing allergens with protein-based methods and, in addition, recoveries are heavily dependent on the applied epitopes of different ELISA kits. The results of MS-based methods (e.g. for isotope-labeled quantification) have to be calculated by a conversion of the raw data, such as the peak area, from the ratio of unlabeled/labeled peptide to the peptide concentration on the column. Subsequently, the peptide concentration in the sample extract can be determined by taking the dilution factors and the recovery of the sample preparation into account. Next, the peptide concentration has to be converted into the target allergenic protein in the sample extract. Considering the relative allergenic protein content in the total protein composition, the total allergenic protein in the sample extract can be calculated. Applying the moisture-corrected sample weight and the extractable protein content, the amount of dry allergenic ingredient can be determined (Parker et al., 2015). In the context of the allergenic potential of peanut, the protein-based techniques normally detect only a small number of proteins whereas 17 allergens are known[c]. Furthermore, the actual determination is merely based on a marker peptide as indicator for the allergenic protein (Holzhauser, 2018). Depending on the applied ELISA kit, and thus depending on the different antibodies, the recovery of the allergenic protein of peanut in muffins (spiked: 5000 ppm dark roasted peanut flour) were 0.2, 4.6, 9.4, 11.7 to 27.1%, with a mean recovery of 10.6% (Parker et al., 2015). It has to be kept in mind, that an additional extrapolation from allergenic protein to the concentration of allergenic food adds a further degree of uncertainty to the results.

In conclusion, the inherent variation of results when applying different ELISA kits and especially the necessary number of post-processing conversions from marker peptides to the allergenic content render the declaration of protein-based methods as direct

[c] http://www.allergen.org/search.php?allergensource=peanut&searchsource=Search

detection applications questionable. On the other hand, DNA-based allergen detection methods require the assumption that a positive result of species-specific DNA sequences correlates with the presence of protein of the food.

Considering the individual thresholds in the underlying clinical studies of the VITAL program, the determination of the reference doses is not based on the individual allergenic proteins, but on the allergenic food itself or the total protein of the allergenic food (Holzhauser, 2018). Taking this into account, the analytical tools have to determine the content of the allergenic food to meet the VITAL requirements or to enable the surveillance of the action values of the German food control. So for both, the protein- and the DNA-based methods, calculations from the actually detected parameters, regardless of DNA sequences, different epitopes or marker peptides, are needed to be able to determine the actual content of the allergenic ingredient. Nevertheless, analytical methods have been developed and are applied for the detection and the quantification of food allergens with both, protein- and DNA-based techniques. As aforementioned, both techniques are necessary to complement each other and allow the analysis of all allergenic components, for which labeling is mandatory.

5.2.2 Protein-based quantification methods

Egg, milk and gluten have to be quantified using protein-based approaches as aforementioned. Various ELISA kits with sensitivities in the low ppm range are commercially available (Schubert-Ullrich et al., 2009). These are applicable for the surveillance of the required action values. Amongst others, different ELISA kits detecting ovomucoid or ovalbumin as main allergens in hen's egg, and kits specific for casein and β-lactoglobulin are commercially available. Especially for gluten analysis the ELISA kit RIDASCREEN® Gliadin (R7001, R-Biopharm, Darmstadt, Germany) in combination with the R5 ELISA RIDASCREEN® Cocktail (R7006/R7016, R-Biopharm, Darmstadt, Germany) for extraction is widely used as actual standard method. It is accepted as AOAC official method of analysis (AOAC, 2012), certified at the AOAC Research Institute (AOAC, 2018), and stated as Codex Alimentarius method (Codex Alimentarius, 1979). The LOD is defined as 1.5 ppm gliadin or 3 ppm gluten, the LOQ is determined to be 2.5 ppm gliadin or 5 ppm gluten (R-Biopharm, 2015). In recent years, MS methods for the detection of various allergens have been developed; five of the first nine published single allergen detection methods are targeting the proteins

αS1-casein, αS2-casein, β-casein, k-casein, BSA and β-lactoglobulin, which indicate the presence of milk, with LODs ranging from 1.25 to 100 ppm milk powder or 1 to 51 ppm target protein (Monaci et al., 2018).

In general, various MS-based methods have been reported that are barely comparable, due to the lack of uniform validation criteria (Monaci et al., 2018). Various methods have been published with different performance criteria that do not allow for a comparison of the methods. Therefore, the AOAC postulates standard method performance requirements for the validation of MS-methods, which aim at the detection and quantification of food allergens[d]. These requirements include the analytical range, LOD, LOQ, recovery and precision. For an accurate validation and to ensure the comparability between the established methods, the reference materials in use, the reporting units including the applied conversion factors from target to allergenic compound per food, and presumably as most important aspect, the impact of the food matrices should be taken into consideration. For the latter, it is not sufficient to mix the extracted and purified allergens with protein extracts of a food, but spiked food should be used.

Considering these prerequisites, only a few methods for multi allergen analysis allow for comparative assessment due to the quality of the presented validation data. For example, Parker et al. (2015) published an MS-based approach for the detection and quantification of whole egg, non-fat dry milk and partially defatted dark roasted peanut flour, which was validated with spiked cereal bars and muffins. The quantification was achieved with stable isotopes, and recovery and variability conversion factors from peptide to total protein of the allergenic ingredient have been presented. The method was further applied to spiked sugar cookies, and the LOD and LOQ were determined (Boo et al., 2018). Sayers et al. (2018) determined lightly roasted mechanically defatted peanut flour in chocolate cookies dessert and chocolate bars and validated the method for recovery, linearity, and sensitivity and also specified the conversion factors from synthetic peptide to total protein of allergenic ingredient. Lyophilized milk powder, whole eggs (isolated egg yolk and egg white), soy flour and peanut butter have been quantified in chocolate, ice cream, tomato sauce and cookies (Planque et al., 2016).

[d] https://www.aoac.org/aoac_prod_imis/AOAC_Docs/SMPRs/SMPR%202016_002.pdf

The method was extended to additionally detect almond, pecan, cashew, hazelnut, walnut and pistachio. Sensitivity, linearity and precision (six aliquots of the same food preparation) have been determined (Planque et al., 2017). A method for the quantification of skimmed milk powder, egg powder, pre-cooked soy flour, hazelnut and peanut has been presented by Pilolli et al. (2017, 2018). Spiked cookies have been used for the validation process and recovery, linearity, LOD and LOQ have been selected as performance criteria. In addition, matrix and processing effects have been considered.

5.2.3 Comparison of MS- and real-time PCR-based quantification of sesame in spiked foods

Huschek et al. (2016) presented a method for the quantification of soy beans, sesame seeds and white lupine with a stable isotope dilution (SID) matrix-matched calibration curve. The study was conducted with spiked cookies and soft bread, and recovery, sensitivity and precision data were provided. Due to the quality of the validation data and the use of spiked matrix material, a comparison on the basis of the performance criteria of this LCMS-based method for the quantification of sesame and the DNA-based approach developed in this thesis (Publication I) is possible (Table 3). The quantification by stable isotope-labeled standard material is a combination of the matrix-adapted quantification and the quantification using internal standard material. An internal multi-level calibration (five levels) is compiled using a weighted linear regression from the analysis of isotope-labeled internal standards. In this approach, the matrix adaption is achieved by adding a defined amount of isotopically labeled peptides to the samples and to the calibration standards (Huschek et al., 2016).

Recoveries obtained by the standard addition method ranged from 72 to 114% of the expected values, which are comparable to the results from the LC/MS method with recoveries from 69.4 to 111.0%. The precision data expressed as relative standard deviations under repeatability conditions (RSD_r) for the real-time PCR based quantification methods ranged from 27.75 to 36.25% (n = 12). In contrast, the precision of the LC/MS based method, also expressed as the relative standard deviation, was determined to be 5.44%. However, it has to be kept in mind, that the determination is based on a lower amount of values (n = 6), and the values are from only one protein

extract. Nevertheless, the low RSD of 5.44% indicates a very precise quantification method.

Table 3: Comparative assessment of the quantification of sesame in spiked foods by LC/MS and real-time PCR

Analytical method		
Detection system	HPLC-triple quadrupole/linear ion trap-(SRM)	Real-time PCR specific for Ses I 1 (2S-albumin of *Sesamum indicum*)
Quantification approach	SID matrix-matched calibration curve	Standard addition
Reference	Huschek et al., 2016	Publication I
Performance criteria		
Recovery [%]	69.4 - 111.0[a]	72 – 114[b]
Precision [%]	5.44[c]	27.75[d]
Sensitivity	20 ppm (in wheat flour)[e] 10 ppm (in cookies)[e] 50 ppm (in soft bread)[e]	2.0 mg/kg (in boiled sausage)[f]

[a] determined from 3 matrices (wheat flour, cookies and soft bread) with 3 spiking levels (50, 75 and 100 ppm)
[b] determined from 1 matrix (boiled sausage) with 4 spiking levels (5, 10, 20 and 100 ppm)
[c] Relative standard deviation, inferred from the values for the determination of the recovery, calculated from 3 matrices (wheat flour, cookies and soft bread) with 3 spiking levels (50, 75 and 100 ppm), but from one protein extract (n = 6)
[d] Relative standard deviation under repeatability conditions (RSD_r): 2 DNA extracts, 4 PCR replicates per extract at day 1 and 2 PCR replicates per extract at day 2, respectively (n = 12) from 4 spiking levels (5, 10, 20 and 100 ppm)
[e] LOQ estimated using a signal-to-noise-ratio of 10
[f] LOQ is defined as the analyte concentration with a relative confidence interval (P = 95%) ≤ 30%

The methods' sensitivities are determined as LOQ. Notwithstanding that they were determined in different ways, the matrix-adapted quantification (2.4 mg/kg) and the quantification by standard addition (1.95 mg/kg) showed lower values than the quantification using an internal standard (6.4 mg/kg). Compared to this, the LOQs of the LC/MS method are higher with 10 mg/kg in cookies, 20 mg/kg in wheat flour and 50 mg/kg in soft bread. On the basis of the value of 11.8 mg/kg given by the VITAL Scientific Expert Panel and the action value of >10 mg/kg of the German food safety authorities the real-time PCR based methods seem to be applicable (Table 2). The LC/MS based method meets the sensitivity needed for the required values only with

the determined LOQ of 10 ppm in cookie. However it has to be kept in mind, that the matrix-adapted quantification does also not reliable quantify in this concentration when the low recovery of 12% is considered. That means the LOQ of 2.4 mg/kg has to be adapted by the factor 8.3 (1/12%) to take the low recovery into account and to calculate the actual amount of detected sesame in the sample material to be 19.9 mg/kg at the limit of detection.

This comparison of the protein- and the DNA-based quantification methods for the determination of sesame indicates different advantages for both methods. Whereas the real-time PCR based approaches indicate a higher sensitivity the LC/MS-based method shows the higher precision.

Protein-based methods are particularly needed in the case of the determination of egg, milk and gluten where DNA-based approaches are not applicable. On the other hand, DNA-based approaches offer the only possibility to determine celery as protein-based methods are inappropriate. Thus, DNA- and protein-based approaches are needed to get reliable results for the optimal risk assessment of potential allergenic foods to give best possible protection to allergic consumer.

5.3 Implementation of the developed methods in the official collection of methods of analysis according to § 64 of the German Food and Feed Act

5.3.1 Standard addition for quantification of soy bean in cereal flour

The standard addition method is very laborious; however, the validation results for the quantification of sesame in spiked food and the simultaneous quantification of soy bean, celery, white mustard and brown mustard showed a promising performance compared to the other DNA-based quantification methods and offer the suitability to screen and quantify four allergens simultaneously (Publication I and II). As a consequence, the quantification by means of standard addition was implemented in the official collection of methods of analysis according to § 64 of the German Food and Feed Act for the quantification of soy bean in cereal flours. The performance criteria of this method have been validated by means of a ring trial organized by the § 64 working group for food allergens with 13 participating laboratories (BVL, 2016).

In the ring trial, soy flour was spiked to wheat and corn flour at concentrations of 100, 500 and 5000 mg/kg. In addition, three of the participating laboratories examined rice cookie material containing soy bean at concentrations of 10, 20 and 100 mg/kg to prove the methods suitability to quantify soy bean amounts less than 100 mg/kg.

The evaluation of the analysis of wheat and corn flour material spiked with soy flour demonstrated the suitability of the method. The recoveries were in a range of 97 to 112% for five of six of the examined samples. Only the corn flour material containing 100 mg/kg soy flour showed a recovery of 145%, however, a deviation of the actual amount of the spiked material was assumed by the organizers of the ring trial. The recoveries of the former published singleplex real-time PCR methods were in a range of 87.4 to 113.7% that confirms the results from the ring trial (Table 4). The mean recoveries obtained applying the tetraplex real-time PCR ranging from 95.1 to 130.0% show a slight overestimation compared to the singleplex approaches. However, the values are still in the range of +/- 50% postulated by Abbott et al. (2010) for trace analysis in this low target concentration range.

Table 4: Comparative assessment of the performance criteria for the quantification of allergenic foods by means of standard addition

Analyte	**Soy bean**	**Sesame**	**Soy bean**	**Celery**	**Brown mustard**	**White mustard**	**Soy bean**	
Analytical approach	Singleplex real-time PCR; Eugster, 2010	Singleplex real-time PCR; Publication I	Tetraplex real-time PCR; Publication II				Singleplex real-time PCR; BVL, 2016	
Quantification range [a] [mg/kg]	100-100,000	1-1000	1-1000				10-10,000	
Concentration range of spiked foods [mg/kg]	800-1500	5-100	40-400				100-5000	10-100
Analyzed matrix	Wheat and rice	Boiled sausage	Boiled sausage				Wheat and corn flour	Rice cookies
Range of recovery [%]	77 - 104	72 – 114	96 – 165	78 – 116	99 – 140	67 – 113	97-145	50-133
Mean recovery [%]	87.4	92	130.0	99.5	124.1	95.1	113.7	92.3
Precision data	Uncertainty from analysis in duplicate	Repeatability (RSD_r)	Repeatability (RSD_r)	Repeatability (RSD_r)	Repeatability (RSD_r)	Repeatability (RSD_r)	Reproducibility (RSD_R)	Reproducibility (RSD_R)
Determined as,		15 – 43	12.0 – 16.2	10.6 – 11.2	4.6 – 8.9	23.7 – 30.0	22 – 31	32 – 110
Range of values [%],	1.4 – 5.3							
Mean value [%]	3.1	28	13.4	10.9	7.3	27.2	24.5	62.0
LOQ [mg/kg]	ND[b]	2.0	8.5	3.7	2.6	36.8	>100	>100

[a] the quantification range is defined by the concentration of the added amounts of the analyte for the standard addition procedure

[b] not determined

The precision data of the ring trial, expressed as the reproducibility (RSD_R), is for all examined samples <35%. In the absence of performance criteria in the field of trace analysis of allergenic foods the criterion from GMO analytics was consulted. There it is stated that the reproducibility should be <35% for the analysis of materials containing more than 0.2% of the analyte. For materials containing less than 0.2% of the analyte a reproducibility of <50% is acceptable (ENGL, 2015). In all cases the more severe criterion is fulfilled. The precision data of the quantification of sesame and of the simultaneous quantification of celery, soy bean, brown mustard and white mustard are expressed as repeatability (RSD_r). Again, the criterion of the GMO analytics is consulted (RSD_r <30%) and is met in all cases, except for the very low sesame concentrations of 1 and 5 mg/kg in boiled sausage.

The quantification of soy bean in rice cookie material results in 50, 133 and 94% for the recovery in the material containing 10, 20 and 100 mg/kg. The reproducibility was determined to be 110, 32 and 44%. On the basis of these results, the authors of the ring trial study came to the conclusion that a reliable quantification is only possible down to a concentration of 100 mg/kg. The recovery and the precision data deviate for the quantification of rice cookie material, compared to the results obtained in the other studies. Therefore, the results have to be critically reviewed.

The quantification range is given by the amount of analyte added for the standard addition. Soy bean in boiled sausage material by means of a tetraplex real-time PCR was quantified by standard addition with a defined quantification range of 1 to 1000 mg/kg. A reliable quantification was proved for materials containing 400, 100 and 40 mg/kg soy bean in boiled sausage material. The limit of quantification was determined to be 8.5 mg/kg which is between the lowest (1 mg/kg) and second lowest (10 mg/kg) amount of analyte added for the standard addition procedure (Publication II). This is confirmed by the quantification of sesame by means of singleplex real-time PCR and the quantification of celery and brown mustard by means of tetraplex real-time PCR. Only for the quantification of white mustard by means of tetraplex real-time PCR, the limit of quantification (between 10 mg/kg and 100 mg/kg) was higher. Considering this, it is not surprising that the quantification down to 10 mg of soy bean in rice cookie in the ring trail failed as the target concentration is equal to the lowest added amount of analyte for the standard addition and thus too near to the limit of quantification. However, considering the results for the quantification of the

material containing 20 mg/kg, a recovery of 133% with a reproducibility of 32% indicates acceptable results even at the theoretical limit of quantification. Taking into account, the precise recoveries from 97 to 112% and the fulfilling of the precision data required for quantitative GMO analysis, this ring trial is a meaningful indication of the applicability of the standard addition for a highly reliable quantification of analyte concentrations from 0.5% to 0.01%. The results of the ring trial confirm the recoveries and the precision data determined in the former studies. These results highlight the suitability of the standard addition method to quantify even traces of allergenic foods with a high trueness and precision. As a consequence, the standard addition has been implemented in the official collection of methods of analysis according to § 64 of the German Food and Feed Act for the quantification of soy bean in cereal flours.

5.3.2 Simultaneous detection and quantification of celery, soy bean, brown mustard and white mustard

The tetraplex real-time PCR method established in this thesis (Publication II) has been validated in an inter-laboratory ring trial (Waiblinger et al., 2017). Spiked boiled sausage material with concentrations of 40 and 100 mg of the allergenic foods per kg and autoclaved sausage material with concentrations of 10, 40 and 100 mg of allergenic foods per kg were analyzed by 13 participating laboratories. The quantification was achieved by a matrix-adapted quantification approach using material containing 400 mg of each allergenic compound per kg sausage material as matrix-matched calibrator.

Functionality of the tetraplex real-time PCR system

The functionality of the tetraplex real-time PCR method was proved by the analysis of a dilution series of DNA of the four analytes with 1000, 316, 100, 31.6 and 10 copies per reaction. From the results of the dilution series of the 13 participating laboratories, the coefficient of variation (R^2) and the slope, and thus the efficiency of the real-time PCR system were calculated for each analyte (Waiblinger et al., 2017; Table 5). In the same manner, the functionality was tested in Publication I by determining R^2 and the efficiency by analyzing a serial dilution of DNA of the four analytes (100 to 0.01 ng per reaction). In accordance with the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009), the assay

performance characteristics PCR efficiency and R^2 of the calibration curves have been reported for each of the real-time PCR systems in the tetraplex assay (Table 5).

Table 5: Functionality of the tetraplex real-time PCR for the detection of soy bean, celery, white mustard and brown mustard determined from DNA dilution series

	Intra-laboratory results from Publication I		Inter-laboratory results from Waiblinger et al. (2017)	
	Efficiency [%]	**R^2**	**Efficiency [%]**	**R^2**
Soy bean	94.8	1.00	82.4	0.99
Celery	94.2	1.00	91.6	0.99
White mustard	90.5	1.00	85.1	0.99
Brown mustard	96.6	1.00	88.2[c]	0.98[a]

[a] Efficiency and R^2 calculated without the values for 10 copies per reaction

For multiplex approaches the slope should range between -3.9 and -2.9, corresponding to efficiencies of 80.5 to 121.2% (Broeders et al., 2014). The intra-laboratory efficiencies determined in this thesis (Publication II) varied between 90.5 and 96.6% whereas the inter-laboratory efficiencies ranged between 82.4 and 91.6%. The coefficient of variation was determined in all cases to be >0.98 which is in compliance with the requirements derived from GMO analytics (European Network of GMO Laboratories (ENGL) working group, 2015).

In addition, the sensitivity of the tetraplex was determined on the basis of DNA copies. To this end, the results for the determination of 10 copies per reaction from the 13 different laboratories were considered and for the determination of soy bean, white mustard and brown mustard 95% of the results were positive. However, it should be mentioned that all negative results for the detection of the 10 copies per reaction of soy bean, white mustard and brown mustard were reported by the same laboratory. For the detection of celery, 100% of the results were positive. These results indicate a sensitivity of 10 copies per reaction (soy bean, white mustard and brown mustard) or even below (celery).

Qualitative analysis by means of tetraplex real-time PCR

The method's suitability to detect traces of soy bean, celery, white mustard and brown mustard in food, was proved by qualitative evaluation of the results of the ring trail. Considering the boiled sausage material, the false positives were 6.4% (soy bean),

5.1% (celery), 5.1% (white mustard) and 2.6% (brown mustard) and the false negatives were 1.3% (soy bean), 0% (celery) and 0% (white mustard). The false negative rate could not be determined for the analysis of brown mustard as the reference material was slightly contaminated.
Evaluation of the results of the autoclaved material revealed that the false negative and false positive rates were increased. However, autoclaving is an extreme heat process that is not expected to be applied in food manufacturing. Remarkable is the fact, that for the detection of brown mustard in the autoclaved material containing 10 ppm no false negatives were determined, indicating a highly sensitive PCR system.

Quantitative analysis by means of tetraplex real-time PCR

The tetraplex real-time PCR was used for quantification of the allergenic foods in two different ways that enables the direct comparison of these two quantification approaches, on the basis of the determined performance criteria (Table 6).

In the ring trail (Waiblinger et al., 2017), a matrix-matched calibrator was employed for quantification of the respective analytes in spiked foods. The C_t values from the samples were matched with a co-analyzed DNA copy based calibration curve to the respective amount of DNA copy numbers. The DNA copy number of the sample was then matrix-matched with an also co-analyzed spiked sausage material with a known amount of analytes. In contrast, for the quantification of the respective allergenic components with the standard addition method (Publication II), no matrix-matching is necessary as the standard addition is directly applied to the analyzed food sample.

The precision data were in the same order of magnitude. The mean repeatability determined for the quantification by standard addition was 14.7% and for the quantification with the matrix-matched calibrator 18.8%. The mean reproducibility of the quantification by matrix-matched calibrator was 30.8% and the mean robustness determined for the quantification by standard addition is 12.4%. That means slightly higher values for the precision data of the quantification with the matrix-matched calibrator; however, the data refer to 14 different laboratories and therefore higher values are expected.

Table 6: Comparative assessment of the performance criteria determined for two quantification approaches via tetraplex real-time PCR combined with matrix-matched calibrator (Waiblinger et al., 2017) and standard addition (Publication II), respectively

	Recovery [%]	Repeatability [%]	Reproducibility/ Robustness [%]	Sensitivity/ LOQ [mg/kg]
Quantification by means of matrix-matched calibrator (Waiblinger et al., 2017)[a]				
Soy bean	137.6	21.3	31.5	< 40[d]
Celery	105.9	22.1	31.6	< 40[d]
White mustard	109.1	16.9	24.4	< 40[d]
Brown/black mustard[b]	158.7	14.8	35.8	< 10[d]
Quantification by means of standard addition (Publication II)[c]				
Soy bean	130.0	13.4	6.9	8.5
Celery	99.5	10.9	8.8	3.7
White mustard	95.1	27.2	18.8	36.8
Brown mustard	124.0	7.3	15.1	2.6

[a] Performance criteria derived from spiked foods (boiled and autoclaved sausages) with spiked amounts of 100 and 40 mg/kg of the respective allergenic ingredient; recovery is determined without results from autoclaved material (Waiblinger et al., 2017)

[b] Brown/black mustard was determined in autoclaved sausages with spiked amounts of 100, 40 and 10 mg/kg and recovery, repeatability and sensitivity was calculated there from (BVL, 2017)

[c] Performance criteria derived from lysate mixtures of boiled sausages with spiked amounts of 400, 100 and 40 mg/kg of the respective allergenic ingredient (Publication II)

[d] Sensitivity was assumed from the analysis of the lowest concentration of spiked material

The validation data of the tetraplex real-time PCR combined with the standard addition using boiled sausages showed that the method is applicable for the simultaneous quantification of soy bean, brown mustard, white mustard and celery. Considering the results from the ring trial using the tetraplex real-time PCR in combination with a matrix-adapted quantification method, the performance criteria prove the established method to provide both, reliable and valid results. While the tetraplex real-time PCR system resulted in recoveries of 95.1 to 130.0%, when using the standard addition method, the combination with the matrix-matched calibrator resulted in recoveries of 105.9 to 158.7% (Waiblinger et al., 2017), although a matrix calibrator similar to the analyzed samples was used for the determination of the respective allergenic components in boiled sausage material. However, when the recovery rates of the autoclaved sausage material are considered, the main disadvantage of the dependency from matrix based calibrators becomes obvious. The

recoveries determined for the autoclaved material containing 100 ppm were 168.3% for brown mustard, 11.7% for white mustard, 10.5% for celery and 33.1% for soy bean. Taking into account, that DNA is highly degraded and thus the recoveries are expected to be lower than 100%, the DNA degradation should be in the same order of magnitude for each of the analyzed analytes. Assuming a mean degradation of 80% (derived from the mean recoveries except 168.3% for brown mustard), and thus a mean recovery of 20%, the recoveries from white mustard (11.7%), celery (10.5%) and soy bean (33.1%) differ from this assumed actual value by 59%, 53% and 166%, respectively.

The established tetraplex real-time PCR has been included in the official collection of methods of analysis according to § 64 of the German Food and Feed Act for the simultaneous detection and determination of black/brown mustard, white mustard, celery and soy bean in boiled sausage material; the quantification is performed with a matrix calibrator (BVL, 2017).

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Publications and presentations

Publications (peer-reviewed)

Luber F., Demmel A., Hosken A., Busch U., Engel K.-H. (2012). Apricot DNA as an Indicator for Persipan: Detection and Quantitation in Marzipan Using Ligation-Dependent Probe Amplification. *Journal of Agricultural and Food Chemistry*, *60* (23), 5853-5858.

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Luber, F., Demmel, A., Herbert, D., Hosken, A., Hupfer, C., Huber, I., Busch, U., & Engel, K.-H. (2014). Comparative assessment of DNA-based approaches for the quantification of food allergens. *Food Chemistry, 160*, 104-111.

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Publications (others)

Luber F., Demmel A., Busch U., Engel K.-H. (2011). Authentizitätsbestimmung von Marzipan: Nachweis von Aprikosen-DNA (Persipan) mittels ligationsabhängiger Sondenamplifizierung. *Lebensmittelchemie, 65*, 127.

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Luber F., Demmel A., Busch U., Engel K.-H. (2011).
Authentizitätsbestimmung von Marzipan: Nachweis von Aprikosen-DNA (Persipan) mittels ligationsabhängiger Sondenamplifizierung.
Presentation at Arbeitstagung des Regionalverbandes Bayern der Lebensmittelchemischen Gesellschaft – Fachgruppe der GDCh, Freising, 15.02.2011.

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