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Detection of peanut allergens by means of new PCR based methods and ELISA

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"The future belongs to those
who believe in the beauty of their dreams"
Eleanor Roosvelt

Questa tesi é dedicata alla mia famiglia
che ogni giorno ha creduto e supportato ogni mio sogno.
Elena

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Contents

Scope 7

Chapter 1 Introduction
- 1.1 What is food allergy? 10
  - 1.1.1 Prevalence of food allergies and the influence of exposure and individual susceptibility factors
- 1.2 What are food allergens? 14
  - 1.2.1 Peanut allergens and peanut allergy
- 1.3 Product safety 17
  - 1.3.1 Legislation concerning food allergens
  - 1.3.2 Food industry and the management of food allergy risk
- 1.4 Detection methods 21
  - 1.4.1 DNA and protein based detection methods
  - 1.4.2 Innovative methods based on Peptide Nucleic Acids
- 1.5 References 30

Chapter 2 Development of three real-time PCR assays to detect peanut allergen residue in processed food products 37

Chapter 3 The effect of heat treatment on the detection of peanut allergens as determined by ELISA and real-time PCR 59

Chapter 4 Peanut allergen detection in chocolate and in products from the market by means of ELISA and real-time PCR 79

Chapter 5 A PNA-Array platform for the detection of hidden allergens in foodstuffs 101

Chapter 6 Unconventional method based on circular dichroism to detect peanut DNA in food by means of a PNA probe and a cyanine dye 113

Chapter 7 Light up probes in real-time PCR for peanut detection 127

Concluding remarks 141

Annex I 143

Acknowledgments 151

Curriculum Vitae 152

Scope

Peanut allergy is an increasingly important public health problem since the ingestion of even low amounts of peanut can trigger severe allergic reactions. Thus it has a strong impact on the quality of life of allergic consumers and their families who have to pay particular attention to avoid products containing peanut traces. To be able to follow such an avoidance strategy they rely on the information provided on the label of foodstuffs and therefore on the efforts of the food industry and food control agencies in assuring the reliability of the labels. The availability of suitable food allergen detection methods is one of the key points in the protection of the allergic consumer since it allows identification of food products that can contain allergenic ingredients. The goal of this thesis is therefore the design and development of new DNA based methods for the detection of peanut allergen residues in real foodstuffs. This design of new methods embraces known techniques like real-time PCR and innovative techniques based on Peptide Nucleic Acid (PNA) probes.

In Chapter 1 a general overview on the problems concerning food allergy as a public health issue is given. Specific legislation regarding the declaration of food allergens on the label of food products is presented and the effort of the food industry in the management of food allergy risks is discussed in this section. The available techniques for the detection of food allergen residues are presented, including new innovative approaches based on PNA.

In Chapter 2 experimental results are presented on the development of three real-time PCR assays for the detection of peanut allergens in foodstuffs. The performances of the three assays are described with regard to their specificity and sensitivity. The application of the techniques for the detection of peanut DNA sequences in a model food matrix is presented.

In Chapter 3 evidence of the effect of heat treatments on the detection of peanut with either the newly developed real-time PCR methods or commercially available ELISA kits is reported. Results on the detection of peanut as impacted by heating of peanut kernels as well as heating of a peanut-containing food matrix are described.

In Chapter 4 experiments are described that demonstrate the extended applicability of the previously developed real-time PCR method to chocolate matrices. Since this real-time PCR method is suitable for the analysis of different matrices that represent two important branches of the confectionary industry (cookies and chocolate) a comparative study between the real-time PCR method and two protein based commercial kits (ELISA and lateral flow device) used for the analysis of two hundred market samples is described. The good agreement between the two different methodologies is described by comparing the analytical results obtained and taking into account the possible effects of the matrix (e.g. cocoa content).

In Chapter 5 the application of PNA combined with PCR is described. Two PNA probes targeting peanut and hazelnut DNA sequences have been combined with microarray technology and the results reported show the feasibility of applying this method to detect traces of these potentially allergenic ingredients in food products.

In Chapter 6 an innovative method for the identification of peanut DNA in food which is based on circular dichroism is reported. The PNAs for post PCR detection of peanut specific DNA is described in combination an achiral 3,3'-diethylthiadicarbocyanine dye (DiSC2(5)). Experimental evidence of the possible application of the optimized method to identify and quantify extracted and PCR amplified peanut DNA from peanut and peanut-containing foods is reported.

In Chapter 7 preliminary results on the possible application of a PNA based probe, a so called Light Up probe, in the real-time PCR detection of peanut are reported. Experimental data are given to show that sensitivity and efficiency are comparable to that of current real-time PCR detection systems but deeper studies are needed to assess and improve the specificity of the new method.

Chapter 1

Introduction

1.1 WHAT IS FOOD ALLERGY?

Food allergies are included within the broad spectrum of food-related illnesses that might be defined as adverse reactions to food. In general adverse reactions to food can affect any individual who consumes food but according to the mechanism provoking the symptoms they can be distinguished in different categories (Figure 1). As a first distinction, they can affect people who do not suffer from any disease related to food, or particular susceptible individuals. The ingestion of a sufficient amount of toxins, microbiological contaminants, or pharmacologically active ingredients can indeed lead to symptoms in everybody. In contrast to this the adverse reactions to foods which only occur in sensitized individuals are defined as food hypersensitivity and only affect a fraction of the population. Food hypersensitivity reactions may either result from psychological factors, that lead to aversion, avoidance and psychological intolerance of a certain food, or from true physical hypersensitivity to food components. When a true hypersensitivity occurs, it can be caused by metabolic abnormality involving an enzyme deficiency (e.g. lactose intolerance) or by a hyper-reactivity to specific substances that are present in food. The last group of food hypersensitivity reactions includes food allergies. Food allergy is defined as "a hypersensitivity reaction initiated by immunologic mechanisms" by the task force of the European Academy of Allergology and Clinical Immunology (EAACI) (Johansson et al., 2001).

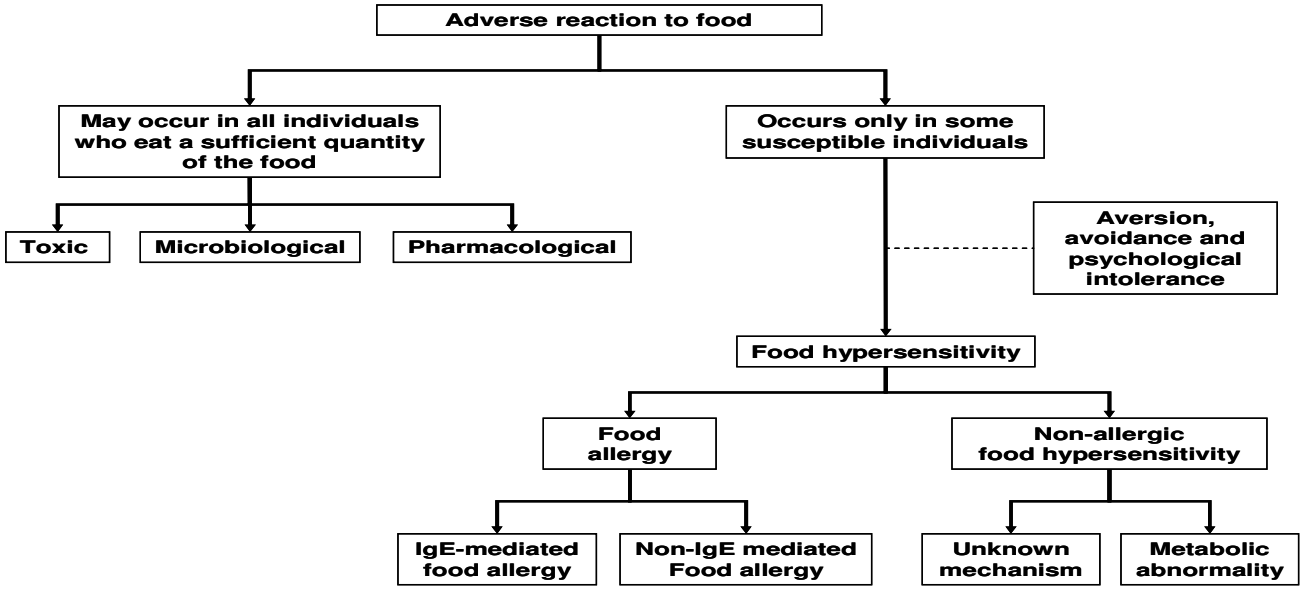

Figure 1: A classification of adverse reactions to food (adapted from Jackson et al., 2003).

Food allergies can be divided into two subcategories according to the mechanism provoking the allergic reaction (Taylor et al., 2001):

- Non-IgE mediated allergies (delayed hypersensitivity reactions) are cell mediated, typically T cell-mediated, allergies in which interactions between cells and chemical mediators, rather than antibodies, are the key factors (Taylor et al., 2001). Symptoms develop hours or even days after exposure to the allergenic food. The delayed reactions can lead to symptoms in different parts of the body such as the skin, the gut and other organs, and probably play an important role when food allergy is a factor in chronic conditions.
- IgE mediated allergies (immediate hypersensitivity) involve the production of antibodies known as immunoglobulin E (IgE) and the establishment of a series of interactions between various cell types and chemical mediators (Taylor and Hefle, 2002). The IgE reaction is immediate and can affect the mouth, the gut, the skin and the respiratory tract. Food allergies involve abnormal immunological responses to specific components of certain foods.

Antibodies (or immunoglobulins) are proteins produced by B type lymphocytes in response to the components that are foreign to the body (known as antigens or allergens). Their normal function is to protect us from parasitic infections. But, in the case of food allergies this mechanism leads to an abnormal immunological response to certain foods in susceptible individuals. Allergens eliciting such an inappropriate IgE formation can be found in food but also in pollen, mold spores, venoms, dust mites and animal danders (Esch et al., 2003).

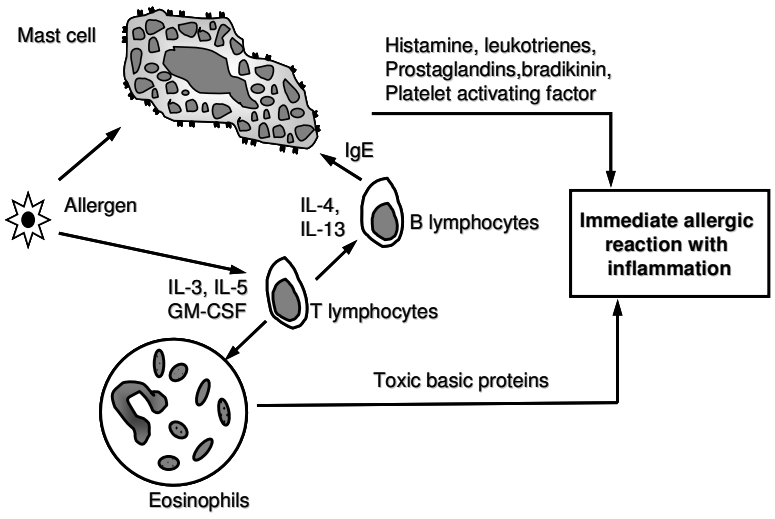

Figure 2: mechanism of an IgE-mediated allergic reaction (adapted from Jackson et al., 2003)

Human antibodies fall into five structural immunoglobulin classes (IgA, IgD, IgE, IgG and IgM), only IgEs are an integral part of the immediate allergic response. IgEs, produced by B lymphocites, have affinity for a specific part of the antigen molecule known as an epitope; the other end of IgE molecules can be bound by immune cells including mast cells. When IgE molecules bind to the mast cell surface, this cell becomes sensitised to the specific allergens that induced the production

of IgE. Once sensitized, exposure to the same food allergens on a subsequent occasion can trigger an allergic reaction (Taylor et al., 2001). The allergen forms a bridge between two IgE molecules on the mast cell surface causing the immediate release of chemical mediators, including histamine, and the release of pro-inflammatory substances, including various leukotrienes and prostaglandins (Figure 2). Other cell types that play a role in the allergic mechanisms are T lymphocites, that can be activated by the presence of the allergen and release mediators. This in turn stimulates B lymphocites to produce more IgE. Other mediators (IL-3, IL-5, GM-CSF, IL-4 and IL-13) activate the local inflammatory process carried out by the eosinophils. The result of the whole process is an immediate allergic reaction accompanied by an inflammation process that can result in localized symptoms at the site of contact (e.g. oral allergy syndrome), localized gastrointestinal allergy with nausea, vomiting or diarrhoea, skin symptoms like urticaria and eczema, respiratory symptoms like rhinitis, systemic anaphylaxis with cardiovascular and gastrointestinal symptoms that sometimes lead to shock (Jackson et al., 2003).

1.1.1 Prevalence of food allergies and the influence of exposure and individual susceptibility factors

The prevalence of food allergy in the general population has been estimated to be around 1-2% in adults and nearly 8 % in children (Sicherer et al., 2003, 2004; Helm et al., 2000; Ortolani et al., 2001). Unfortunately food allergy appears to be an increasing phenomenon with peanut allergy being of particular concern. A recent study highlighted a clear increase of the prevalence of peanut allergy in young children (Hourihane et al., 2007).

Moreover, the prevalence of people who claim to suffer from some kind of food allergy reaches 30 % in Europe (Mills, van Ree IFR), while in another study 25 % of all adults claim to believe that their children are afflicted with a food allergy (Sampson, 2005).

The frequency with which food allergies affects people within the total population remains an estimate because of a lack of precise data. Diagnostic criteria, like a correct distinction between immunological and non-immunological hypersensitivity are few examples of difficulties which prevent a proper evaluation of prevalence level. Regarding the diagnosis of real food allergy, the double-blind placebo controlled food challenge (DBPCFC) is currently the gold standard. Nevertheless other diagnostic criteria, ranging from medical history, diet diaries, positive skin prick test or IgE test are also applied (Sampson, 1999b). In recent years an enormous improvement has been made on the characterization of many food allergens and on the general understanding on adverse reaction to food (Sampson, 2004).

Although further studies are still necessary to define rules governing the prevalence of allergy, particular factors that appear to be important are health conditions, genetic predisposition and exposure.

Exposure factors that can influence the prevalence of food allergies are the amount of allergens consumed, the decreased or increased allergenicity due to the food processing, the possible presence of cross reacting allergens. Exposure is considered an important determinant for the development of food allergy as demonstrated by a different prevalence of sensitivity to certain foods according to the age. In the US cows' milk and eggs represent common allergenic foods for infants and young children who often consume those foods, (Sampson and McCaskill, 1985) while crustacean shellfish and fish are among the most common allergenic foods for adults (Sicherer et al., 1999, 2004). Another example of different exposure to the allergens is based on the eating habits. It has been noticed that in an area where a certain food is commonly consumed, the risk of developing an allergy to that food will be larger than in areas where the consumption of the particular food is more rare. Fish is considered one of the most common food allergens in Nordic countries (Dannaeus A and Inganãs M, 1981) while peanut allergy is high in the USA because of the high consumption (Beyer et al., 2001).

Food processing and preparation may also affect the allergenicity. For example boiling peanut results in a decrease of its allergenicity while roasting increases the allergenic potential (Maleki et al., 2004). This is proposed to explain the lower prevalence of peanut allergy in China where peanuts are mainly boiled or fried compared to the United States where they are consumed roasted (Beyer et al., 2001).

Finally cross reacting allergens represent another important factor for food allergy. Exposure to pollen can induce respiratory allergy to pollen, but because of cross reacting allergens food allergy can also be induced as a result of pollen sensitisation. This is the case of ragweed pollen and melons (watermelon, cantaloupe, honeydew), mugwort pollen and celery, and birch pollen and various foods such as carrots, apples, hazelnuts, and potatoes (Van Ree and Aalberse, 1993; Ballmer-Weber et al., 2000; Enberg et al., 1987; Eriksson, 1986). Although the true incidence and prevalence of food-allergic diseases is not always precisely defined, it is clear that food allergy can affect the lives of a considerable number of people and knowing the factors regulating this phenomenon could contribute to protection of allergic people.

1.2 WHAT ARE FOOD ALLERGENS?

Among the enormous variety of foods that form the human diet only a few are responsible for the occurrence of allergic reactions. Food allergenicity is due to the presence of certain components of the food that constitute the allergen repertoire. Although the vast majority of food allergens are proteins, only a few of the numerous proteins present in foods are known to be allergens (Taylor, 2002).

Sensitization to food allergens can occur in the gastrointestinal tract (class 1 food allergy) or via inhalant allergens (class 2 food allergy). The majority of food allergens provoking a class 1 food allergy are proteins or gliyco-proteins with molecular weights ranging from 10 to 70 kDa. They are usually quite stable to heat, acid and protease treatment (Sampson, 1999a). Allergenic proteins can have very different biological properties: some are storage proteins, some are transport proteins or regulatory proteins and enzymatically active proteins.

Most of the plant allergens are found in the cupin and prolamin superfamilies, or function in the plant defense system. The cupin superfamily consists of the 7S (vicilins, such as Ara h 1, Jug r 2, Ses i 3) and 11S (legumins, such as Ara h 3, Cor a 9, and Ber e 2) seed storage proteins. The prolamin superfamily consists of cysteine-rich 2S albumin storage proteins (eg, Ara h 2, Jug r 1, Ber e 1, and Ses i 2), nonspecific lipid transfer proteins (eg, Cor a 8, Mal d 3, and Pru av 3), and cereal a-amylase and protease inhibitors. Many proteins generated by the plant defense system have been found to be major allergens.

The allergenicity of each single protein is due to its IgE-binding epitopes. Depending on their structure, two kinds of epitopes are described (Figure 3).

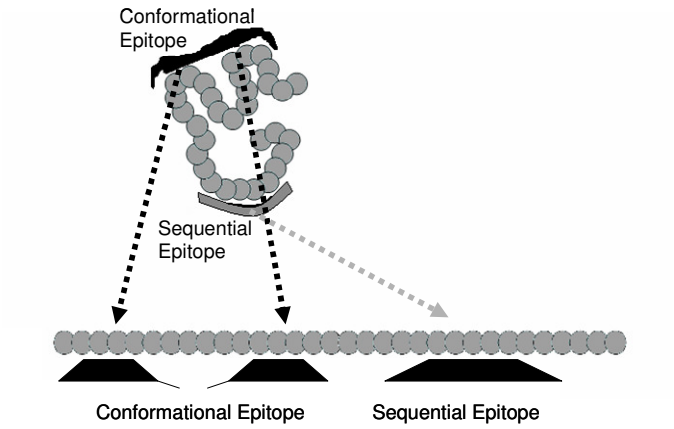

Figure 3: sequential and conformational epitopes. Conformational epitopes are destroyed when the native structure of a protein is modified by e.g processing, whereas sequential epitopes are not affected (adapted from Sampson, 2004)

i) Sequential epitopes that are composed of short peptide fragments (12-18 amino acids) are associated with the linear sequence of amino acid residues. They are believed to be responsible for food allergies, that persist after processing because of their heat-stable epitopes. ii) Conformational epitopes are associated with the 3-dimensional structure of the protein and are usually displayed on the surface area of the molecule. In general, the stability of these epitopes to any type of food processing or digestion is strongly associated with the native protein structure.

Previous studies have shown that individuals who possess IgE antibodies to sequential epitopes react to the food in any form (eg, extensively cooked or partially hydrolyzed), whereas those with IgE antibodies primarily to conformational epitopes appear to tolerate (small amounts) of the food after extensive heating or partial hydrolysis because the tertiary structure of the protein is altered and the conformational epitopes are destroyed (Urisu et al., 1997; Yamada et al., 2000).

The sensitivity of food allergy sufferers to specific food allergens varies widely between individuals. In some cases very small amounts of the allergenic component can trigger an allergic reaction, whereas in other cases less severe reactions occur after exposure to much higher doses. This variability makes it difficult to estimate the lowest dose of a food allergen that is likely to provoke an adverse reaction.

The notion of determining threshold levels for allergenic foods below which sensitised consumers are not at risk of developing allergic reactions has attracted much attention from regulatory bodies, consumer associations and industry throughout Europe. The best estimates of the no observed adverse effect level (NOAEL) for allergic reactions are based on the results of experimental double-blind food challenge studies but also for this experimental approach many variables can affect the results. Such variables include the severity of the allergic condition, the symptoms used as the clinical read-out system (subjective vs objective symptoms and their associated severity), the different administration protocols, the challenge conditions and food preparations, the allergen content and matrix of challenge foods, the total amount of administered dose and time frame, reproducibility (false positives and negatives), the effects of co-factors (for example exercise, alcohol, medication), the patient population (different geographical distribution of underlying sensitisation rates for cross-reacting allergens) and on individual's ethnicity.

The setting of minimal eliciting doses for various allergenic foods is further complicated by the fact that for individual food allergic patients the minimal eliciting doses vary by several orders of magnitude (Taylor et al., 2002c; Hourihane et al., 1997) and symptoms and eliciting doses can change over time for each individual. Thus, any value for NOAEL obtained will not necessarily represent all people in the population that are allergic to the same food. For example, the minimal

eliciting doses for peanut that can provoke mild adverse reactions in a group of peanut allergic individuals range from 2 to over 50 mg (Hourihane et al., 1997).

1.2.1 Peanut allergens and peanut allergy

Peanuts are among the most allergenic foods known. The International Union of Immunological Societies Nomenclature Subcommittee recognizes 8 allergenic proteins in peanuts, from Ara h 1 to Ara h 8. Among these 8 proteins, Ara h 3 and Ara h 4 are nearly identical isoforms and Ara h 6 is highly homologous to Ara h 2 (Koppelman et al., 2003; de Leon et al., 2007). The 3 major allergens, Ara h 1-3, are comprised of vicilin, conglutin, and glycinin seed storage proteins, respectively (de Leon et al., 2007). Two of the 8 identified peanut allergens, Ara h 5 and Ara h 8, are not storage proteins but are implicated with pollen-associated food allergy and are a profilin and a Bet v 1–like protein respectively (Mittag et al., 2004). The allergenic proteins have a high abundance in peanut. Peanut contains around 29% protein and the major allergen Ara h 1 accounts for approximately 20% of this total protein content, while Ara h 2 accounts for around 10% (van Hengel et al., 2007a). Moreover, Ara h 1 and 2 show resistance to heat and enzymatic digestion (Burks et al., 1998).

Because of the high allergenic potential of peanut, peanut allergy has become a real public health problem, attracting the attention of food control agencies, food industry and the scientific community. Peanut allergy is typically life long and sensitive individuals can occur in symptoms ranging from a mild urticaria to life threatening anaphylaxis (Yunginger et al., 1988; Bock and Atkins, 1990). Food anaphylaxis fatalities registries reported peanut as the cause for most of the reported deaths attributed to food allergies over the last 5-7 years (Bock et al., 2007; Pumphrey and Gowland, 2007). Moreover in the USA and in Europe recent studies showed that peanut allergy is an increasing phenomenon especially among children. The latest estimates of the prevalence levels of peanut allergy for children are in the region of 1% (Sicherer et al., 2003; Grundy et al., 2002; Hourihane et al., 2007). It has been estimated that for only 20% of young children by school age food allergy resolves (Hourihane et al., 1998; Skolnick et al., 2001).

Prevalence and epidemiologic characteristic of peanut allergy might be explained by general genetic and environmental factors that are already known to influence food allergy.

The prevalence of peanut allergy varies between countries and seems to be related to consumption measured on a per capita basis. In China for example, peanut allergy prevalence is significantly lower than in the United States (Beyer et al., 2001). The protein composition of various peanut species from around the world has been studied and found to be rather constant (Koppelman et al.,

2001). But, a number of factors related to harvesting and processing of peanuts may have a significant effect on the allergenic properties of various peanut products. Studies on the processing of peanut have shown a major effect on the amount of extractable protein and, in particular after roasting, peanuts have shown increase in allergenicity compared to boiling or frying (Beyer et al., 2001; Hinds et al., 1997).

Since peanut is a widely used in food preparation and pre-packed foodstuffs, allergic patients are often exposed to peanut allergens and unfortunately, the annual incidence rate of accidental ingestion of peanut was found to be 14.3% among schoolchildren in Montreal, Quebec, Canada (Yu et al., 2006). Trace amounts of undeclared peanut present in food products can be hazardous to peanut allergic individuals. Most studies have shown that low amounts of peanut protein (1-3 mg) are sufficient to trigger the objective allergic symptoms (Taylor et al., 2002c; Morisset et al., 2003) while only 200 µg can be sufficient to elicit a mild, subjective allergic reaction (Tariq et al., 1996; Wensing et al., 2002). Given the high allergenicity, the incurable nature of food allergy and its potential life-threatening consequences, the management of food allergy relies heavily on a strict avoidance diet that has to be implemented by food allergic individuals and their families. These food and social restrictions have consequently a strong impact in the quality of life of allergic consumers and their care givers.

1.3 PRODUCT SAFETY

1.3.1 Legislation concerning food allergens

World-wide legislative initiatives have been aimed at regulating food products labelling with particular concern for food allergens.

Within the European Union, a fundamental document on the protection of food consumers is the White Paper on Food Safety, presented by the Commission in 2000 (European Commision, 2000). It assures the European citizen that having the highest standards of food safety is a key policy priority for the Commission. An important issue dealt with in this document is the concept of traceability throughout the feed and food chain: at every level of the production flow, from raw material down to the supermarket shelf. Furthermore adequate records should be kept to trace the origin of a certain product at any time and in particular to withdraw any feed and food from the market whenever a risk to the health of consumers can be envisaged. In accordance to this strategy food labelling legislation has been implemented in order to provide consumers with clear and detailed information on the composition of the foods they eat.

Directive 2000/13/EC describes which type of information is required on the label of food products (European Parliament and Council, 2000). With regard to food allergens, this Directive contains a list (Annex IIIa) naming twelve major allergenic foods whose presence in foodstuffs has to be declared on the label of such food products (cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk, tree nuts, celery, mustard, sesame and sulphites). A new Directive, 2003/89/EC, was introduced as an amendment of Directive 2000/ 13/EC and abolishes "25 % rule" for compound ingredients, ensuring that the components of ingredients forming less than 25 % of the final product are indicated on the label in order to guarantee that all ingredients should be declared on the labels, regardless of the quantity contained in the finished food (European Parliament and Council, 2003). Food labelling requirements concerning food allergens were also modified in order to ensure that derogations to the obligatory declaration of food ingredients were not applicable to those ingredients (listed in Annex IIIa) that may trigger food allergic reactions. More recently, Commission Directive 2006/142/EC announced the inclusion of lupin and molluscs (European Parliament and Council, 2006) into the list in Annex IIIa of Directive2003/89/EC. The above mentioned directives only refer to allergenic ingredients that are known to be used in the production (and present in a finished food product) and do not provide threshold levels below which food products are exempt from the labelling requirements (except for sulphur dioxide and sulphites, where such a threshold level is set at 10 mg/l). Therefore and the accidental presence of allergenic ingredients in food products is not covered. In 2005, Directive 2005/26/EC (European Parliament and Council, 2005) established a list of food compounds that were provisionally excluded from the labelling requirement because it was considered that they were not likely, to trigger adverse reactions (e.g. wheat base glucose syrup including dextrose, whey or nuts used in distillates for spirits, mustard seeds and oil). The provisional exceptions that were granted expired on 25 November 2007. Therefore, after analysis of requests for permanent exemptions from the labelling according to Annex IIIa, a new directive (2007/68/EC) was introduce to update and finalize this annex (European Parliament and Council, 2007). For specific products a permanent exemption was accepted (e.g. whey or nuts used in distillates for spirits are permanently exempted but not mustard seeds and oil).

In order to cover with the legislation also the possible inclusion of allergens in food products resulting from adventitious contamination Directive 2001/95/ EC on product safety (European Parliament and Council, 2001) plus Regulation 2002/178/EC on food safety (European Parliament and Council, 2002) have to be considered. Foodstuffs containing allergenic ingredients not indicated on the label are therefore considered unsafe for a specific category of consumers (consumers with a food allergy) and, consequently, should not be placed on the market.

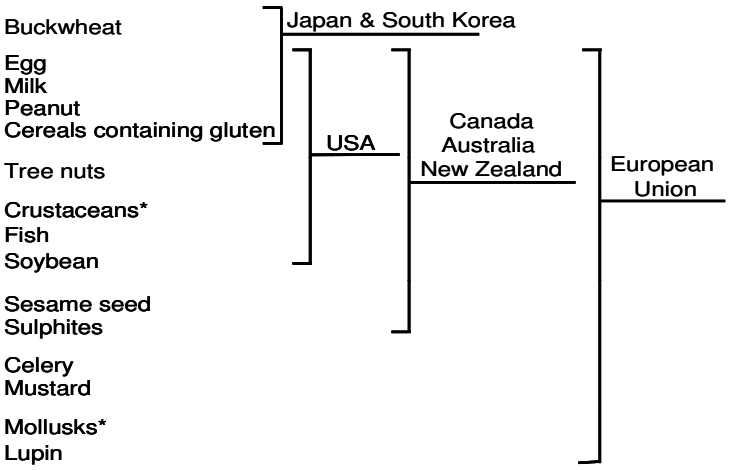

Figure 4: Food allergens that currently need to be declared on the label of packaged foods, *In the USA and Canada crustaceans are grouped with shellfish and therefore include several types of molluscs. In Australia, New Zealand and the EU crustaceans do not include molluscs (adapted from van Hengel, 2007).

Food allergy is considered a worldwide health problem and as Figure 4 shows, many countries have legislative tools in place that require mandatory labelling of certain allergenic foods. For example in the USA, a new labelling law, the Food Allergen Labelling & Consumer Protection Act (FALCPA), was introduced at the start of 2006 giving a clear definition of "major allergen" and listing the "Big 8 allergens" that are required to be labelled (Food Allergen Labelling and Consumer Protection Act, 2004).

1.3.2 Food industry and the management of food allergy risk

New legislations in many countries concerning food labelling, now require the mandatory declaration of specific allergenic ingredients in the manufactured products. However, risks of the presence of food allergens are not only associated to the use of that specific food as ingredient; allergenic residues can be present through cross-contact during manufacturing or because of their presence in raw materials.

Food allergen risk management aimed at the protection of sensitized consumers. This can lead to the use of precautionary labelling or the implementation of specific management risks for food allergens. From a manufactures point of view this means taking actions in order to reduce the chance that allergens unintentionally end up in food products.

Guaranteeing the total absence of such constituents from a product for which they are not used as ingredients is often practically impossible or quite expensive for the food industry. Manufactures and retailers, therefore resort to the use of precautionary warnings (eg. "may contain peanut" or "this product is made in a factory that also produces peanut-containing products"). Allergic individuals are often unsure about the risk posed by food products carrying precautionary labels. Furthermore their frequent use considerably reduces the food choices while it does not clearly

assure presence of absence of the offending food. On the other side, by heeding the advice of precautionary labels allergic consumers put themselves at risks (Hefle et al., 2007; van Hengel, 2007b).

A better approach would be the analysis of the risk arising from residual allergens and subsequently a quantitative risk assessment. The process of risk assessment is conventionally divided into four separate stages: hazard identification, hazard characterization, exposure assessment and risk characterization (Codex Alimentarius Commission, 2003).

In the case of food allergy the hazard under consideration is any adverse reaction to food mediated by the immune system, namely food allergic reactions.

The second step is the hazard characterization which consists of establishing the relationship between the triggering dose and the response it produces. Food allergens differ from materials for which a conventional toxicological risk assessment can be made because they do not provoke reactions in the general population. Food allergens trigger the reaction to a subset of the population but not at a similar dose ingested. Not accurate data is available with regard to the highest dose that does not elicit an allergic reaction (NOAEL), since sufferers react differently to a defined dose and the relationship between dose and severity varies between individuals. Once the hazard has been characterised and a NOAEL can be defined, exposure assessment would be required. The usual measure used for purposes of risk assessment is the amount of allergen that can be present in a portion of food, although other issues should also be considered like the period of intake of allergen or the possibility of cross-reactions.

Practical measures that have to be taken in risk management need an integrated approach along all stages of production. Such measures can include the selection of non-allergenic ingredients in innovative products, the control of allergens in the supply chain, an inclusion of allergens in the HACCP plans (Hazard Analysis of Critical Control Points), the implementation of correct labelling that ensures that appropriate allergen information is made available to the consumer.

Along the phases of the production chain, a number of points might be identified where detection of allergenic residues could provide valuable information required to assess the risk arising from the inadvertent presence of the allergenic ingredient. If defined requirements are not met, particular procedure like special cleaning should be applied (Crevel et al., 2007, Hefle and Koppelman, 2006).

1.4 DETECTION METHODS

Methods for the detection of allergenic residues are required for a variety of purposes. In food industry, detection methods can be included in the HACCP plan in order to assess the extent of cross contact at different points of the food production chain or for investigation in case of accidental presence of the allergenic residues in foodstuffs. Public authorities represent another user. They need to check for compliance with legislation with regard to the presence of allergen traces in products that are not supposed to contain them. Different users might require different methods with different characteristics, with respect to detection limits, quantitation, robustness and ease-of-use.

Various food allergens detection methodologies already exist and are based on diverse technologies and can be designed for different purposes. The target molecule represents the first main distinction being either protein or DNA. Detecting proteins is the most common approach since this directly detects the molecules responsible for triggering allergic reactions. However, detection of allergenic proteins or marker proteins is not necessarily the only way to demonstrate the presence of the allergic compound, and the detection of another type of marker molecule like DNA can be an alternative. The most common methods that are currently employed for the detection of food allergens are listed in section 1.4.1. Section 1.4.2 deals with innovative DNA based methods that can be employed to detect food allergens by utilizing Peptide Nucleic Acids (PNAs).

1.4.1 DNA and protein based detection methods

Protein based detection methods:

- RAST/EAST inhibition : RAST (radio-allergosorbent) or EAST (enzyme-allergosorbent) assays are *in vitro* assays, designed for the detection of allergen-specific human IgE antibody. They are both based on the use of human sera from allergic patients and are mainly applied in the diagnosis of food allergy (Holgate et al., 2001). In addition to their clinical application, protocols of RAST and EAST inhibition tests have been adapted to detect and quantify the residual food allergens in acqueous extract of a wide range of foodstuffs (Nordlee et al., 1981 Oldaeus et al., 1991). RAST and EAST inhibition analyses are based on a competitive binding of human IgE. The principle of the methods is based on an allergen which is bound to a solid phase. This allergen functions as an antigen for specific human IgE that can bind to it. This binding is inhibited by free antigen/allergen present in the sample solution. Subsequent detection of bound IgE is based on anti-IgE antibodies labelled with an isotope (RAST) e.g. ^{125}I, or an

enzyme (EAST). The quantitative nature of this inhibition permits an assessment of the amount of allergen in the extract. Although the limit of detection of RAST was shown to be around 1 mg per kg (Fremontet al., 1996; Koppelman et al., 1999), standardization and commercialization of this assays is prevented by the limited availability of human serum and its high variability (Nordlee et al., 1995).

- Immunoblotting: One-dimensional sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting represents the standard procedure to separate proteins and identify allergens. After separation according to their molecular mass the proteins are transferred from the gel onto a membrane and detected with radio- or enzyme-labelled antibodies. This method has allowed the detection, identification and characterization of a large number of individual allergens (Pastorello and Trambaioli, 2001). Detection of allergenic molecules in food products is another area where this SDS PAGE followed by immunoblotting can be applied. Detection limits (LOD) that can be achieved with this application are in the region of 5 mg/kg (Scheibe et al., 2001). The major disadvantages of SDS-PAGE and immunoblotting is the reliance on IgE from human sera which can be quite variable and might cross-react with other non specific-food ingredients. However, after an allergen has been characterized, human IgE can be replaced by antibodies raised in animals.

- Dot immunoblotting: This represents a simpler and less expensive way to screen food samples for the presence of food allergens. The detection procedure is identical to the one described above but the sample is directly spotted onto a membrane without any pre-separation of the proteins. The intensity of the dot is proportional to the amount of antigen/allergen, which allows a semi quantitative detection of the target protein or mixture of proteins (e.g. peanut) in food. Reported LODs are in the region of 2.5 mg/kg (Blais and Philippe, 2001). A disadvantage of this method is the possibility of false positive results because of cross-reactivity of the antibodies with matrix components.

- Rocket immuno-electrophoresis: This is an analytical method based on a gel containing antibodies. Sample proteins migrate according to their electrophoretic mobility and when antigen-antibody complexes form in the gel, this leads to the formation of precipitates in the shape of a rocket. Since the formation of such complexes will only take place at a constant antigen/antibody ratio, . the height of the rocket is proportional to the amount of antigen in the sample.

Applications of rocket immuno-electrophoresis in the detection of allergens in various food products have been reported (Malmheden Yman et al., 1994; Holzhauser et al., 1998) with LODs ranging from 2.5 to 30 mg/kg. Rocket immuno-electrophoresis is not used widely because of the laborious gel preparation and immuno-staining procedures.

- ELISA: Enzyme-linked immunosorbent assay (ELISA) is probably the method that is used most commonly by the food industry and official food control agencies. In the food industry, ELISA tests are usually used to detect antigens such as allergens, pesticides, mycotoxins or pathogens in a sample. This test is based on the use of an enzyme linked to an antibody to detect the formation of the complex between antigen and antibody. The enzyme produces a colorimetric reaction and a standard curve generated with the use of allergen standards allergen with known concentrations allows (semi)quantification of allergens in food products.

  Two types of ELISA systems are employed for the detection of food allergens, competitive ELISA and sandwich ELISA (Figure 5). Almost all commercial allergen ELISA test kits use the sandwich technique.

  Sandwich ELISA: A capture antibody is immobilized on a solid phase, which is usually a microtiter plate or a multiple well strip, and it specifically binds (allergenic) proteins. A second protein-specific antibody labelled with an enzyme responsible for a colorimetric reaction, detects the first antigen-antibody complex. The concentration of the antigen/allergen is proportional to the colour intensity, which can be measured with a spectrophotometer. Sandwich ELISA methods have been developed for the detection of several different food allergens (Koppelman et al., 1999; Mäkinen-Kiljunen and Palosuo, 1992; Hefle et al., 1994; Holzhauser et al., 1999) and numerous commercial test kits have become available during the last decade.

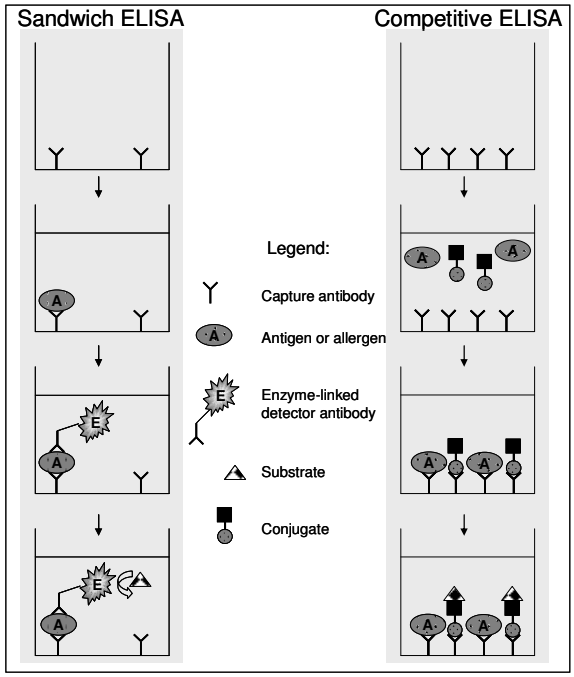

Figure 5: on the left mechanism in a typical sandwich ELISA; on the right, steps of a competitive ELISA.

  Competitive ELISA: This type of ELISA competitive operate on the basis of competition between the horseradish peroxidase (HRP) enzyme conjugate and the antigen in the sample for a limited number of specific binding sites fixed on the precoated microplate. The bound enzyme

conjugate is detected by the addition of a substrate which generates a colorimetric reaction. Therefore the absorbance is inversely proportional to the concentration of antigen in the sample. This technique is often preferred for the detection of relatively small proteins. Competitive ELISA systems have been described for the detection of several food allergens (Koppelman et al., 1999; Hlywka et al., 2000; Mariager et al., 1994; Plebani et al., 1997; Yeung and Collins, 1996).

Recent validation studies (Whitaker et al., 2005; Park et al., 2005; Poms et al., 2005) have shown that all ELISA test kits studied were capable of correctly identifying test samples containing 5 mg peanut per kg food matrix.

However, variables like the type of test kit, the food matrix, the spiking method, the material used for spiking, as well as food processing methods can affect the detection of allergenic ingredients in food products (Whitaker et al., 2005; Park et al., 2005; Poms et al., 2005; Koch et al., 2003).

- Dipsticks: Lateral flow immunochromatographic assays, commonly known as dipsticks are fast and easy to use devices that can also be used to detect the presence of allergens in food in non-research settings. This methodology is also based on an immunological detection of proteins that are captured by specific antibodies, conjugated to coloured particles. The success of this methodology is based on the fast flow of the antibody-antigen complex along a test strip (nitrocellulose or nylon). The complex is captured by a zone of antibodies specific for the antigen along the strip, which results in the development of a visible line. Dipsticks are used as qualitative methods, although the intensity of the band which is correlated to the concentration of antigen in the sample, suggesting a limited potential for (semi) quantification.

  A validation of 2 study of types of commercially available dipsticks in which 18 laboratories participated has shown that the LOD of those products lies between 5-20 mg/kg (van Hengel et al., 2006). Therefore, the sensitivity of dipsticks is higher than the one that can be achieved with ELISA test kits.

<u>DNA based detection methods:</u>

- PCR: Polymerase Chain Reaction (PCR) -based methods are characterized by three consecutive steps. Firstly the DNA is extracted and purified, then specific DNA sequences are amplified and finally the amplified products (amplicons) are detected. This approach is currently used for the detection of microbial pathogens (Malorny et al., 2003), genetically modified crops in food products (Anklam et al., 2002, Holst-Jensen et al., 2003) and food allergens in food products

(Poms et al., 2004). In food allergens detection, the target DNA is species-specific and functions as a marker for the presence of a particular food ingredient.

The invention of the PCR in 1983 is attributed to Dr. Kary Mullis. It involves an enzymatic amplification of nucleic acid sequences via repeated cycles of DNA denaturation, oligonucleotides annealing and DNA polymerase extension. The oligonucleotides or primers can anneal to complementary single stranded DNA obtained by heat denaturation of double-stranded DNA. The DNA polymerase enzyme can add extra nucleotides to the primer by using the genomic DNA as a template. Subsequent heat denaturation and annealing of the second primer to the newly synthesised single-strand DNA allows synthesis of a complementary DNA strand. After several cycles the amplified product can be visualised in the following manners:

By gel electrophoresis: This only provides information on the size of the amplified product but does not reveal the identity of the PCR product.

By Southern blotting: There the amplified product is detected after hybridization to a labelled version of the target DNA which provides a means for identification of the amplified product.

In general PCR results are qualitative and only DNA sequencing allows a complete identification of a PCR product. A (semi-)quantitative approach can be achieved by two more recent PCR methodologies: PCR-ELISA or real-time PCR.

- PCR-ELISA: after PCR amplification, the products are detected with an ELISA protocol. The amplified DNA is labelled either by the use of a modified primer that will allow binding of the DNA to coated microtiter plates, or by the incorporation of labelled nucleotides (e.g. DIG labelled UTP). In the first case, binding of the DNA is followed by denaturation and subsequent hybridization with a labelled DNA probe. Enzyme-linked antibodies are then used to detect the target DNA (which is present as a complex of amplified target DNA – labelled probe – enzyme-linked antibody). When labelled nucleotides are incorporated during PCR, the DNA can be bound to a solid phase and enzyme-linked antibodies capable of binding the labelled nucleotides are then used for detection. The colour development that is driven by the enzymatic reaction can be measured and provides a way for semi-quantification of the target DNA. This method combines the advantages of PCR and ELISA, but the combination of the two techniques makes it also more laborious and time-consuming.

- Real-time PCR: This is based on the measurement of a fluorescent signal that increases during the amplification of PCR products. Most of the commonly used real-time PCR assays are based

on Taqman chemistry (Figure 6). The tube in which the PCR reaction takes place contains a target-specific oligonucleotide probe with a fluorescent reporter dye and a quencher attached to it. The proximity of the quencher to the dye prevents the detection of fluorescence. But, when the probe hybridizes to the amplified target DNA, the 5' exonuclease activity of the polymerase cleaves the probe and thereby separates the quencher from the dye which is displaced by the newly synthesised DNA strand. The fluorescence of the free reporter dye can then be measured and it is proportional to the amount of amplified products. The sigmoid curve obtained when the fluorescence level is plotted against the number of amplification cycles is used to quantify the target DNA present in the sample before the reaction. Quantification is based on the so-called threshold cycle (Ct), the PCR cycle at which the fluorescent signal can be distinguished from the background noise. Real-time PCR methods allow the detection of allergenic ingredients in food products at a level of 10 $mgkg^{-1}$ or lower (Stephan and Vieths, 2004; Hird et al., 2003; Scaravelli et al., *in press*). However, the robustness and sensitivity of the method remains to be proven in proper method validation trials.

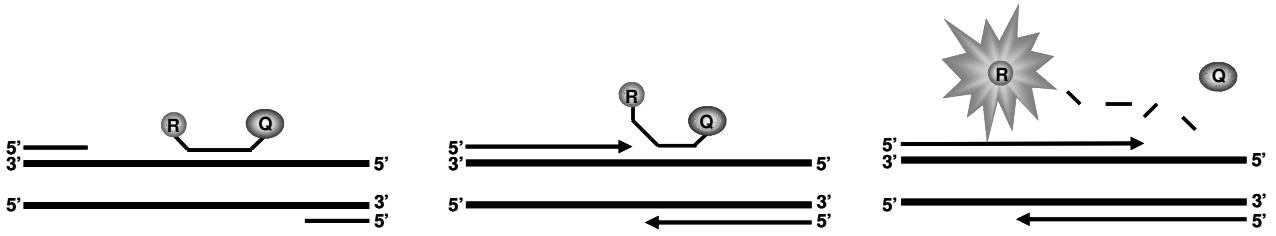

Figure 6: steps in real-time PCR based on TaqMan probes. 1: annealing of primers and probe but reporter signal is quenched by the quencher (Q) when the probe is intact; 2: displacement of the probe by DNA polymerase activity; 3: cleveage of the probe and generation of the reporter (R) signal (adapted from Applied Biosystem)

Biosensors: Biosensors are analytical devices that consist of a biological recognition element (e.g. cells, proteins, oligonucleotides) that are in direct contact with a sensor chip. Upon contact with target molecules a signal is generated which is further processed to give an output that is proportional to the concentration of a specific analyte. The advantages of this technology are the short analysis time and the high degree of automation. The sensitivity of this method largely depends on the characteristics of the sensor chip. LODs in the range of 0.45 to 2.0 have been described for the detection of milk proteins in food products (Muller et al., 2004; Indyk et al., 2004). In addition to this, biosensors can be used to discriminate between intact and degraded protein/allergen (Muller et al., 2004; Dupont et al., 2004).

Proteomic approach: the proteome is the collection of all protein components present in a complex system. Most food allergens are components of a proteome and since they are usually glycoproteins,

post translational processing like glycosylation does affect the allergen proteome. Proteomic research is usually based on separation of proteins and the identification of individual proteins. Many improvements in separation and identification of proteins, such as two-dimensional electrophoresis, nano-liquid chromatography and mass spectrometry, have rapidly been achieved (Hirano et al., 2004). These techniques have enabled a high throughput analysis of complex protein mixtures. At present proteomic research is mainly focused on the identification of allergens (Natale et al., 2004), but it is expected to be used to study allergen modifications and allergen detection in food products. No clear data on the limit of detection of allergens are currently available.

1.4.2 Innovative methods based on Peptide Nuclei Acids (PNAs)

Peptide Nucleic Acids (PNAs), are synthetic achiral oligonucleotide analogues in which the sugar phosphate backbone is replaced by a polyamide chain covalently linked to the nucleobases (Nielsen et al., 1991). More in details, the negatively charged sugar phosphate backbone is replaced in the PNA structure by a neutral pseudo-peptide N-(2-aminoethyl)glycine units linked by amide bonds and the four nucleobases (i.e. adenine, cytosine, guanine and thymine) are linked to the backbone via methylene carbonyl linkages at equal distance as in DNA bases. Thus, PNA contains the same number of backbone bonds between bases (i.e. six) and the same number of bonds between the backbone and the bases (i.e. three), as in DNA. The synthesis of PNA oligomers follows the established synthetic procedure commonly employed for the peptide synthesis.

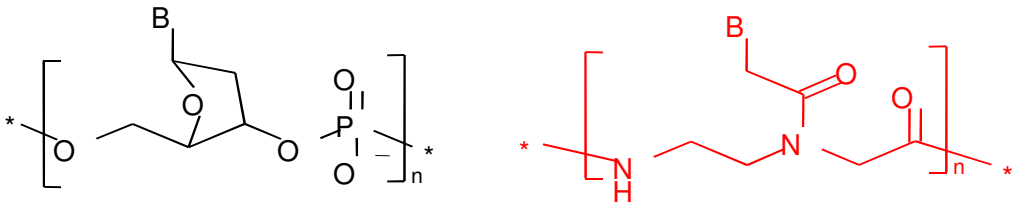

Figure 7: Chemical structure of DNA and PNA

Thanks to its unique structure PNAs offer higher affinity and specificity in recognizing and hybridizing DNA sequences as compared to DNA-DNA duplexes:

- PNA-DNA duplexes show a higher thermal stability than DNA-DNAes duplex: the hybridization in this case obeys the Watson-Crick rules through formation of hydrogen bonds (Egholm et al., 1993) and it is not affected by the electrostatic repulsion which normally occurs during hybridization between the negatively charged molecules of DNA. This higher thermal

stability leads to an average increase of the melting temperatures of 1°C per base pair, compared to the corresponding DNA/DNA duplex;

- PNA-DNA hybridization is independent from the salt concentration of the hybridization solution (Orum et al., 1995), thanks to the neutral backbone of PNA;
- thermal stability of the PNA/DNA duplexes is strongly affected by the presence of mismatches. The presence of one single mismatch destabilizes the PNA/DNA duplexes much more than the corresponding DNA/DNA duplexes making the PNA more sequence selective for specifically recognising its complementary sequence (Hyrup et al., 1996);
- PNA-DNA orientation (Figure 8): parallel and antiparallel orientations of binding may occur, but antiparallel binding is preferred on account of its higher thermal stability. PNA can bind a target double stranded DNA in other different ways: triplex formation, duplex invasion and triplex invasion. In particular triplex structures produce a great increase in the melting temperature of the PNA/DNA/PNA complexes (a T_{10} PNA can bind an A_{10} DNA forming a triplex with Tm=72°C);

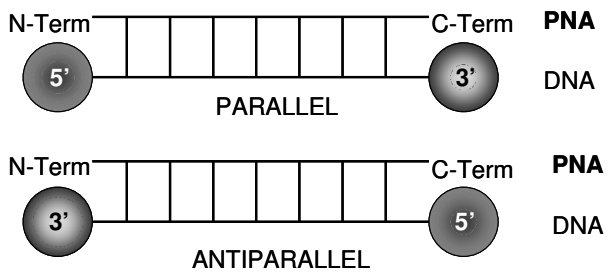

Figure 8: Parallel and antiparallel orientation of the PNA/DNA duplexes

- high biological stability: PNA does not undergo degradation by nucleases and proteases

Due to its hybridization properties, PNA has been used in many biomedical and diagnostic applications.

As for the biomedical applications, the ability of PNA in selectively hybridizing with high stability not only DNA but also RNA complementary sequences has been successfully employed in antisense strategies to block the translation of specific mRNA into proteins. A large number of diagnostic applications have been developed so far employing PNAs in combination with other analytical methods, in combination with PCR methods, in PCR clamping and in real-time PCR experiments (Kyger et al., 1998). The so-called light-up probes, consisting of a PNA oligonucleotide linked to an asymmetric cyanine dye, has recently been developed for sequence specific detection of DNA from *Salmonella* via post PCR analysis and from *Yersinia enterocolitica* via real-time PCR (Wolffs et al., 2001; Isacsson et al., 2000). Moreover, in clinical diagnostics some commercial kits for qualitative and quantitative detection of viral DNA (cytomegalovirus and SARS coronavirus) based on the combination of LightUp probe Technology and PCR are already

available (ReSSQ® Assays, LightUp probe Technologies, Huddinge, Sweden, http://www.lightup.se/). PNA has been also successfully incorporated in molecular beacons for the detection of specific DNA target sequences (Kuhn et al., 2001, 2002) and in GMO detection combined with HPLC technology (Totsingan et al., *in press*). A molecular beacon consists of a hairpin loop structure, where the loop portion is a probe sequence that is complementary to a target sequence in a nucleic acid. A reporter fluorophore is attached to one end and a quencher to the other: in the hairpin loop structure the molecular beacon is not fluorescent whereas, upon hybridization to a complementary structure, the fluorophore and the quencher are separated, giving rise to a fluorescent signal.

The incorporation of PNA in light up or molecular beacons probes results in higher hybridization efficiency and specificity and they can be applied in real-time PCR or for post-PCR analysis thanks to a lower influence of the salt concentration and to their stability to nuclease and protease.

Sequence identification with PNA probes are also employed in MALDI-TOF mass spectrometry (Griffin et al., 1997) and capillary electrophoresis (Igloi, 1999). Several PNA based biosensors have been also developed and employed for different applications (Wang, 1998): among these electrochemical biosensors, quartz crystal microbalance (QCM) transducers and surface plasmon resonance (SPR) spectroscopy were the most successful.

Methods for genotyping single nucleotide polymorphisms (SNPs) are important in many biomedical applications and especially for prediction of hereditary diseases; PNAs in this case have been employed in combination with single-stranded DNA specific nucleases (i.e. nuclease S1). The DNA/PNA duplexes with a mismatch are hydrolyzed by this nuclease, whereas fully-matching sequences are kept intact. This difference is visualized by adding 3,3'-diethylthiadicarbocyanine, which changes its colour from blue to purple upon binding to DNA/PNA duplexes (Komiyama et al., 2003; Wilhelmsson et al., 2002).

Food diagnosis is also one of the field in which PNA probes have been employed.

The detection of a single molecule of DNA coming from transgenic maize was achieved with a new laser based fluorescence technique utilizing PNA probes (Castro et al., 1997). Rapid detection, identification and enumeration of microbial contamination in bottled water have been achieved with the use of PNAs to develop a new chemiluminescent in situ hybridization (CISH) method (Stender et al., 2000). Moreover PNAs have been applied to the detection of specific sequences related to GMO and food allergens analysis by its combined use with HPLC (Lesignoli et al., 2001; Germini et al., 2005a).

Another approach in food analysis which has been recently developed with the combined use of PNAs is the microarray technology. The microarray system consists of immobilized probes

spatially located on planar surfaces, microchannels, microwells or arrays of beads which are then hybridized with the target molecule (fluorescently or radioactively labelled).

Array based systems have been employed so far for studying the biochemical properties and activities of target proteins printed on the array, the interactions with other proteins, nucleic acids, lipids, small molecules and other biomolecules. In addition, DNA microarrays have been produced with synthetic oligonucleotides, PCR products or cDNAs in order to implement expression studies or mini-sequencing reactions. In order to improve the sensitivity and reduce time consuming, PNAs have been substituted to common oligonucleotides in GMO detection or food authenticity by post-PCR analysis (Germini et al., 2004, 2005b). Furthermore, recent studies highlighted the possibility to reveal DNA hybrid formation by label-free detection using both MALDI-TOF (van den Boom et al., 2004; Edwards et al., 2005) and Surface Plasmon Resonance techniques (Su et al., 2005; Spadavecchia et al., 2005).

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Chapter 2

Development of three real-time PCR assays to detect peanut allergen residue in processed food products

Hypersensitivity to peanut is a public health problem, since the ingestion of even low amounts of peanut can trigger severe allergic reactions. Allergic consumers rely on the information provided on the label of foodstuffs to identify products that might endanger their health. In order to protect the allergic consumer methods are required for the detection of allergenic ingredients. For this purpose we have developed three real-time polymerase chain reaction (PCR) assays, based on TaqMan chemistry, that are capable of detecting peanut specific DNA sequences in food products. The peanut specific sequence targeted for detection is located within the gene family of the allergen Ara h 3. The occurrence of multiple Ara h 3 sequences in the peanut genome increases the chance to achieve a good sensitivity. DNA extraction is also known to affect detection by PCR, therefore the efficiency of several different DNA extraction methods was compared.

The methods reported here are capable of detecting 2.5 pg peanut DNA (less than one copy of peanut genome equivalent) and all three assays were successfully applied to detect peanut traces in a model food product where they could detect 10 mg kg^{-1} peanut.

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Introduction

Food allergies might be defined as adverse, immune-mediated (IgE mediated) reactions to foods that can occur in sensitized individuals. The prevalence of IgE-mediated food allergies is not precisely known, but is estimated to be around 4 % of the general population (Sicherer et al., 2004). Amongst infants and children food allergy occurs even more frequently with prevalence levels reaching 8 % (Helm and Burks, 2000). The severity of the reactions varies from mild urticaria to potentially lethal anaphylactic shocks. Common allergenic foods include cow's milk, crustaceans, hen's egg, fish, tree nuts, peanuts, soy and wheat (FAO, 1995).

Peanut allergy is one of the most severe food allergies due to its persistency throughout the lifetime of individuals and its life threatening character, even after the ingestion of minimal doses (100 µg of peanut protein) (Hourihane et al., 1997a, 1997b). It is estimated that peanut and tree nut allergies affect 1.1 % of the US population (Sicherer et al., 1999) and according to a recent study, the prevalence of peanut allergy in children has doubled between 1999 and 2003 (Sicherer et al., 2003).

People suffering from a food allergy usually try to avoid consuming the food that can endanger their health. Despite this, the ingestion of peanuts often happens accidentally because of mislabelling of products, rework processes which include peanut-containing foods or cross-contamination during processing (Sampson et al., 1992; Yunginger et al., 1988). For a better protection of allergic consumers and to ensure compliance with new European legislation resulting from Directives 2000/13/EC and 2003/89/EC on food labelling (European Parliament and Council, 2000,2003), detection methods are required to specifically detect the presence of hidden allergens in a wide variety of food items.

Usually food allergens, the components capable of triggering allergic reactions, are proteins or glycoproteins, but only a small fraction of all food proteins are allergenic. Several studies have found that the major peanut allergens are resistant to digestion and food processing techniques (Burks et al., 1998), while roasting has been shown to increase the allergenicity of this food (Maleki et al., 2000).

Enzyme linked immunosorbent assays (ELISA) (Besler, 2001) are most commonly used to determine the presence of allergenic ingredients like peanut in food products. Immuno-detection based methods employ antibodies raised in animals against peanut proteins. Some of these kits are designed to specifically recognize one allergenic protein (Krska et al., 2004), while others detect a pool of proteins contained in the protein extract of the allergenic food. According to a recent study the sensitivity of commercial ELISA test kits varies with some test kits being capable of detecting concentrations below 5 mg per kg of food matrix (Poms et al., 2005). Other ELISA methods have

been reported to detect 0.2-1.2 mg peanut per kg food matrix (Kiening et al., 2005). The efficiency, specificity and sensitivity of ELISA are directly related to the potential of the antibodies to recognize peanut proteins. However, the detection of peanut proteins is compromised by the fact that the food industry utilizes a vast number of different ingredients and furthermore it employs a variety of food processing methodologies like heat treatment or roasting which can modify, denature and degrade food proteins (Immer, 2006). Modifications of protein structure and matrix effects might interfere with protein extraction or antibody binding (Crevel, 2006) which can give rise to false positive or false negative results. It is therefore important to have a variety of methods at hand to improve the specificity and to limit false negative and false positive results. An alternative to the direct detection of proteins is the determination of species-specific DNA originating from the allergenic food by means of polymerase chain reaction (PCR). PCR is already routinely applied for the detection and quantification of genetically modified organisms (GMOs) (Anklam et al., 2002). In real-time PCR, DNA amplification measurements are based on the detection of a fluorescent signal that proportionally increases during the generation of PCR products. A threshold line is set at the level of detection at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the Cycle Threshold, Ct. The target sequence can be either designed on the gene coding for the allergenic protein or simply, on a species-specific region in the genome of the allergenic food. Despite their different targets, ELISA and real-time PCR are often designed for quantitative purposes. A comparison of a real-time PCR method and an ELISA, for the detection of peanut has recently been reported, whereby the relative Ct values obtained by the real-time PCR correlated well with the amount of peanut measured by the sandwich ELISA (Stephan and Vieths, 2004). This result highlights the potential of PCR for a semi-quantitative detection of peanut traces in foods. Two real-time PCR assays have been published and the reported limits of detection were in the range of those that can be obtained by means of ELISA, around 1 or 2 mg kg^{-1} (Stephan and Vieths, 2004; Hird et al., 2003). Commercial PCR based kits for the detection of peanuts are currently also available, either in the format of a real- time PCR kit, a PCR combined with ELISA detection, or conventional PCR followed by gel electrophoresis analysis. Such products usually claim to be able to detect 10 mg kg^{-1} peanut in food products. However, food processing often negatively impacts the detection, and therefore an effort should be made to develop methodology that is capable to detect allergenic ingredients in the low mg per kg range (Poms et al., 2004), even in processed foods.

Here we report the development and optimization of three real-time PCR assays based on Taqman chemistry. All three methods were designed to target sequences coding for the peanut allergen Ara

h 3, which belongs to the glycinin family (Koppelman et al., 2003). The peanut genome contains multiple Ara h 3 sequences and since the detection of a multicopy gene allows an improvement of the sensitivity (Dahinden et al., 2001) we have chosen this gene family to design PCR amplicons. A simultaneous development of three different methods, based on different primer pairs, was aimed at identifying the most sensitive method amongst the three. Since the performance of the methods also depends on the quality of the DNA extracted from the samples, we compared 15 different extraction methods in order to optimise DNA extraction and to achieve low detection limits.

Materials and Methods

DNA extraction methods

Fifteen extraction methods were investigated for the isolation of peanut DNA. Raw (Chinese Red skin) and roasted peanuts (obtained from Migros, Zurich, Switzerland) without shell were ground under liquid nitrogen using a mortar and pestle. A "blank of extraction", sample which undergoes the complete extraction procedure without any DNA containing material (peanut), was included in all analyses.

DNA was extracted using five different commercial kits according to the manufacturers' instructions: **i)** DNA extraction kit (GMO & Allergen; Tepnel Biosystems, UK); **ii)** DNAMITE Plant Kit extraction (Microzone Limited, UK); Wizard® Magnetic DNA Purification System for Food (Promega, Madison, USA) **iii)** using 100 mg or **iv)** 1 g of starting material; **v)** GenElute ™ Plant Genomic DNA Kit (Sigma, USA); **vi)** Easy-DNA™ Kit for genomic DNA isolation (Invitrogen Ltd, UK). Furthermore, two commercial kits were used with slight modifications to the manufacturers' protocol in order to increase the DNA yield: **vii)** SureFood® PREP Allergen Kit for preparation of DNA from food products (R-Biopharm / Congen, Germany): 250 mg of samples were weighted out and incubated at 65 ºC for 3 hours or overnight instead of 1 hour to lyse the cells; **viii)** Nucleon PhytoPure plant and fungal DNA extraction kit (Amersham Biosciences Corp.,USA): where the incubation for the DNA extraction after addition of Reagent 2 was prolonged from 10 minutes to 30 minutes or 1 hour. Centrifugation steps were carried out at 9 000 g for 5 minutes instead of 5 000 g for 5 minutes.

Traditional extraction methods were also employed. **ix)** CTAB (Cetyl Trimethyl Ammonium Bromide) extraction: sample aliquots of 100 mg were incubated for 1 hour at 65 °C with 10 ml extraction buffer (2% CTAB, 1.4 M NaCl,; 0.1 M Tris/HCl; 20 mM EDTA; pH 8). After

incubation, the samples were centrifuged for 13 minutes at 9383 g, the supernatant was transferred to a new 50 ml capped centrifuge tube, mixed with an equal volume of chloroform, vortexed and centrifuged again for 13 minutes at 9383 g. The aqueous phase was mixed with two volumes of precipitation buffer (0.5% CTAB; 0.04M NaCl, pH 8) and incubated overnight at room temperature in the dark. The samples were centrifuged for 13 minutes at 9383 g, the supernatant was discarded and the pellets were redissolved in 1 ml 1.2 M NaCl, vortexed and transferred to a 2 ml reaction tube and mixed with 400 µl Cl_3CH. After centrifugation (10 minutes at 12 000 g) the upper aqueous phase was transferred to a new 2 ml reaction tube and 0.6 volumes isopropanol was added plus 0.1 volumes 3 M sodium acetate. The samples were incubated for 10 minutes at -20 ºC, after which they were centrifuged at 10 000 g for 10 minutes, the supernatant was discarded and the pellet redissolved in 500 µl 70% ethanol. The samples were centrifuged once more for 10 minutes at 12 000 g, the supernatant was carefully pipetted off and the pellets were dried at room temperature for 1 hour. The pellets were resuspended with 100 µl TE buffer (10 mM Tris; 1 mM EDTA, pH 8).

x) The extraction method based on a protocol described by Aljanabi (Salah and Aljanabi, 1997). It was applied to 1 g of ground sample (this required scaling up all volumes of reagents required in the protocol by a factor 10). **xi)** In order to improve the yield of extraction RNase digestion and lipid removal with chlorophorm was included: 1 g of ground sample was mixed with 4 ml extraction buffer (0.4 M NaCl, 10 mM Tris-HCl, 2mM EDTA; pH 8), 400 µl of SDS 20% and 15 µl of Proteinase K (20 mg/ml). The samples were incubated at 60 °C overnight, after which 3 ml of 6 M NaCl was added to each sample. Samples were vortexed for 30 seconds at maximum speed and tubes spun down for 30 minutes at 9 500 rpm. To the supernatant 10 µl RNAse (100 mg/ml) was added and the digestion was carried out at 40 °C for 30 minutes. An equal volume of cold $CHCl_3$ was then added to each sample; each sample was vortexed at maximum speed and centrifuged 5 minutes at 9 500 rpm, at 4 °C. The supernatant was then transferred to new 2 ml tubes and 1 ml of isopropanol was added to each tube; the samples were incubated 2 hours at –20 °C. All the samples were centrifuged at 12 000 rpm, at 4 °C for 20 minutes. The pellets were washed twice with 70% ethanol, dried and finally resuspended in 500 µl of water.

Traditional extraction methods were in some cases combined with commercial kits for DNA purification: **xii)** CTAB extraction buffer was combined with the SureFood® PREP Allergen Kit for the purification of the DNA from the slurry.

DNA extraction was also performed combining a guanidine hydrochloride based extraction buffer (Germini et al., 2005) and different commercial kits for DNA purification from the resultant slurry. The DNA was purified from 0.5 – 1 ml of slurry using the **xiii)** DNeasy Plant Mini Kit (Qiagen

Belgium/Luxembourg), **xiv)** SureFood® PREP Allergen kit or **xv)** Wizard® DNA Clean up system (Promega, Madison, USA) according to the manufacturers' instructions.
After the investigation of the most suitable extraction method, only method **xv)** was used for all the samples analyzed. Samples of 2 g (± 0.1 g) were extracted in duplicate and eluted in 100 µl.

DNA quantification

Quant-iT™ PicoGreen® dsDNA regent (Invitrogen Ltd, UK) was utilized for DNA quantification. Quantification was carried out using a Perkin Elmer Reader Victor 1420-040 (Perkin Elmer, USA), according to the instructions of the Quant-iT™ PicoGreen® dsDNA regent kit protocol for a high range standard curve (from 10^{-3} to 1 ng/µl) in a final volume of 200 µl.

PCR methods

Commercial PCR kits

Aliquots of 5 µl DNA extracts from raw and roasted peanut as well as from cookies containing peanuts obtained with the extraction methods described above, were analyzed with the following two different commercial kits designed to detect peanut traces in food products. The BioKits Allergen DNA Selection Module PCR (Tepnel, UK) is a PCR kit combined with gel electrophoresis as post-PCR. The experiments were carried out according to the protocol of the product utilizing AmpliTaq® Gold DNA polymerase (Applied Biosystems, UK) as suggested by the manufacturer and using an iCycler® Thermal Cycler instrument (Bio-Rad, USA).
The SureFood® Allergen Peanut real-time PCR Kit (Congen / R-Biopharm, Germany) was also used according to the protocol of the product and the experiments were carried out on an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, UK).

Conventional control PCR

Amplification of different non-coding regions of chloroplast DNA (Taberlet et al., 1991) was performed in order to avoid false negative results by confirming the presence of vegetable amplifiable DNA. DNA extracted by means of the **xv)** procedure was analysed using PCR Reagent System (Invitrogen Ltd, UK). The concentrations of the reagents in the final volume of 25 µl were: 1 x reaction buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 0.1 u/µl of Invitrogen Taq, and 100 ng of DNA or 5 µl DNA extracts. The reaction was run on an iCycler® Thermal

Cycler (Bio-Rad, USA) and the temperatures applied were as follow: 95 °C for 3 minutes; 40 cycles of 94 °C for 45 seconds, 60 °C for 30 seconds and 72 °C for 1 minute; followed by one step at 72 °C for 10 minutes. The PCR products were analysed on a 2 % agarose gel in 0.5 x TBE.

Real-time PCR

Primer and probe design

Three sets of primer pairs and probes were designed on the target sequences of Ara h 3 genes. DNA sequences (AF093541, AF510854, AY848698, AF125192, AY722685, AY722686, AY722687, AY618460, AY722688, AF086821, AY439332 and AF487543) were obtained from on line available databases (http://www.ncbi.nlm.nih.gov/) and specific tools for DNA alignment and software were employed to identify conserved and specific regions (http://www.ebi.ac.uk/clustalw/, http://www.ncbi.nlm.nih.gov/BLAST/). The probes were 5' labelled with 6-carboxyfluorescein (FAM) and 3' labelled with the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA). For the design of primers and probes the following software was tested: Primer Express (Applied Biosystems, UK), Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), Integrated DNA Technology (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx), mfold (version 3.2, Zuker and Turner (http://www.bioinfo.rpi.edu/applications/mfold/dna/form1.cgi)). Finally all primers and probes were designed by hand and purchased from Applied Biosystems, Warrington, UK.

For PCR A (amplicon of 78 bp) the following primers and probe were designed: forward primer "A forward" 5'- CGC AAA GTC AGC CTA GAC AA -3', reverse primer "AB reverse" 5'- CTT GTC CTG CTC GTT CTC T -3' and probe "AB probe" 5'- FAM-TGC TGT CCT CGA GGG CTA AAT TCA CGC TCT TC-TAMRA -3'. For PCR B (amplicon of 105 bp) the following primers and probe were designed: forward primer "B forward" 5'-GAA GCT TAC CAT ATA GCC CAT ACA-3' "AB reverse" (see above) and probe "AB probe" (see above). For PCR C (amplicon of 114 bp) the following primers and probe were designed: forward primer "C forward" 5'- TGT TTG TCC CTC ACT ACA ACA -3', reverse primer "C reverse" 5'- GAA GCT CCT CGT CGT ACA -3' and probe "C probe" 5'- FAM-ACT TGC ACG TGA GCC CGT CCC CTC AAT -TAMRA -3'.

Real-time PCR conditions

To optimise the performance of the PCR reactions the following parameters were tested: primer concentrations (0.05, 0.3 and 0.9 µM), annealing temperatures (53, 55, 56, 60 °C), elongation

temperatures (60, 72 °C), PCR mastermixes (TaqMan® Universal PCR Master Mix, Applied Biosystems, UK; JumpStart™ Taq ReadyMix™ with dUTP, Sigma, USA; HotStartTaq Master Mix Kit, Qiagen GmbH, Germany; Platinum® PCR SuperMix High Fidelity, Invitrogen Ltd, UK). The optimum assay was set as follows: primer concentration of 0.9 µM, probe concentration 0.25 µM, final volume of 25 µl, PCR mastermix Taqman Universal Master Mix (Applied Biosystems, UK). The thermal cycling was set as follows: a UNG digestion step of 2 minutes at 50 °C, DNA denaturation and Taq Polymerase activation at 95 °C for 10 min followed by 40 cycles of DNA denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds and elongation at 72 °C for 30 seconds. Acquisition of the signal took place during annealing and elongation of each cycle.
All reactions were run on the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, UK).
For sample analysis two separate DNA extractions were performed and amplified in two separate reactions. All results were analyzed by setting the automatic baseline of the instrument and 0.15 as threshold. The Ct values obtained and the relative standard deviations in this paper refer to the mean value of the four replicates of amplification. On each 96-well plate three non template controls (NTCs) and two positive samples were used as controls.

Specificity Analyses

The specificity of the three assays was determined by testing the amplification of non-peanut DNA extracts from a wide variety of raw foods (Table 1). The extraction was carried out according to method **xv)**. In order to avoid false negative results, the amplificability of DNA extracts from vegetable matrices was tested with the conventional control PCR. The three real-time PCR assays (PCR A, B and C) were then applied to all the DNA extracts according to the developed protocols. Each amplification was carried out in duplicate, adding 5 µl of DNA extract (20 ng / µl). In case of non vegetable matrix one more replicate of amplification was added to the real-time PCR plate which was spiked with 1 µl of pure peanut extract (in order to check the presence of possible inhibitors of the PCR reaction).

Preparation of a model processed food matrix

Cookies were prepared in collaboration with the Federal Research Centre for Nutrition and Food, the Institute for Cereal Potato and Starch Technology in Detmold, Germany.

A finely ground peanut powder consisting of equal aliquots of the 5 peanut components included in the IRMM-481 peanut test material (IRMM, Belgium) was prepared by mixing and grounding in liquid nitrogen with an Ultraturrax to obtain a particle size < 250µm. The peanut powder was used to prepare a series of spiked butter mixtures.

100 g of peanut powder was mixed with a blender in 2000 g butter (Süßrahmbutter Lipischer 82% fat) at 35 °C for 2 minutes. Then 200 g of the peanut/butter mix was used to prepare the next lower concentration (1/10) by mixing it with 1800 g blank butter. This dilution step with blank butter was repeated 3 more times.

Aliquots of 1800 g peanut-butter mix were subsequently used to prepare the cookie dough by mixing it with 5000 g wheat flour, 200 g blank butter, 1875 g dust sugar, 600 g skimmed milk powder, 30 g sodium chloride, 12.5 g sodium hydrogen carbonate, 15 g ammonium bisulphate, and 675 g water.

In addition to this, blank cookie dough was prepared in which the peanut-spiked butter was replaced by blank butter. The blank (peanut free) and the five spiked doughs prepared as described above were aliquotted in cookie moulds and baked for 16 minutes at 180 °C (Miwe Condo oven).

The final peanut content in the cookies was approximately 10 000, 1000, 100, 10, and 1 mg kg^{-1}

All the spiked and blank cookies were ground separately, sieved on 0.7 µm sieves and the resulting powder was vacuum packed in aluminium foil bags in aliquots of 15 or 30 g, and stored at -20 °C until use.

Results and discussion

Primer and probe design

Ara h 3 is a peanut allergen that undergoes extensive proteolytic processing resulting in a protein pattern composed of multiple bands as visualized by SDS PAGE analysis. In addition to this, it has been shown that Ara h 3 has several isoforms that are encoded by at least 5 genes (Piersma et al., 2005). Since the similarity with Ara h 4 reaches 88 %, Ara h 3 and Ara h 4 are considered to be isoallergens (Viquez et al., 2004; Yan et al., 2005). From the NCBI DNA data base (http://www.ncbi.nlm.nih.gov/), we retrieved 12 (full or partial) peanut sequences with high homology to Ara h 3 as encoded by AF093541, being AF510854 (Ara h 3/4), AY848698, AF125192, AY722685, AY722686, AY722687, AY618460, AY722688, AF086821 (Ara h 4), AY439332 and AF487543. Since targeting sequences in conserved regions of the multicopy gene

Ara h 3 is likely to improve the sensitivity of detection methods, primers and probes were designed to be specific to conserved areas within the Ara h 3 gene family. Figure 1 displays an alignment of 8 sequences focussing on regions in which the sequences showed a high homology. In order to design assays that specifically detect peanut DNA, primers and probes were designed in DNA regions that were unique to peanut and lacked similarity, with all non-peanut sequences present in the data base. Specific software like Integrated DNA Technology, Primer Express and Primer 3, were employed to check if the parameters for primer and probe design could be met. Unfortunately due to the strictly defined settings this software was found to be not particularly suited for our primer and probe design since targeting the selected conserved regions of Ara h 3 limited the freedom of design and thereby impacted the requirements for real-time PCR conditions and specificity.

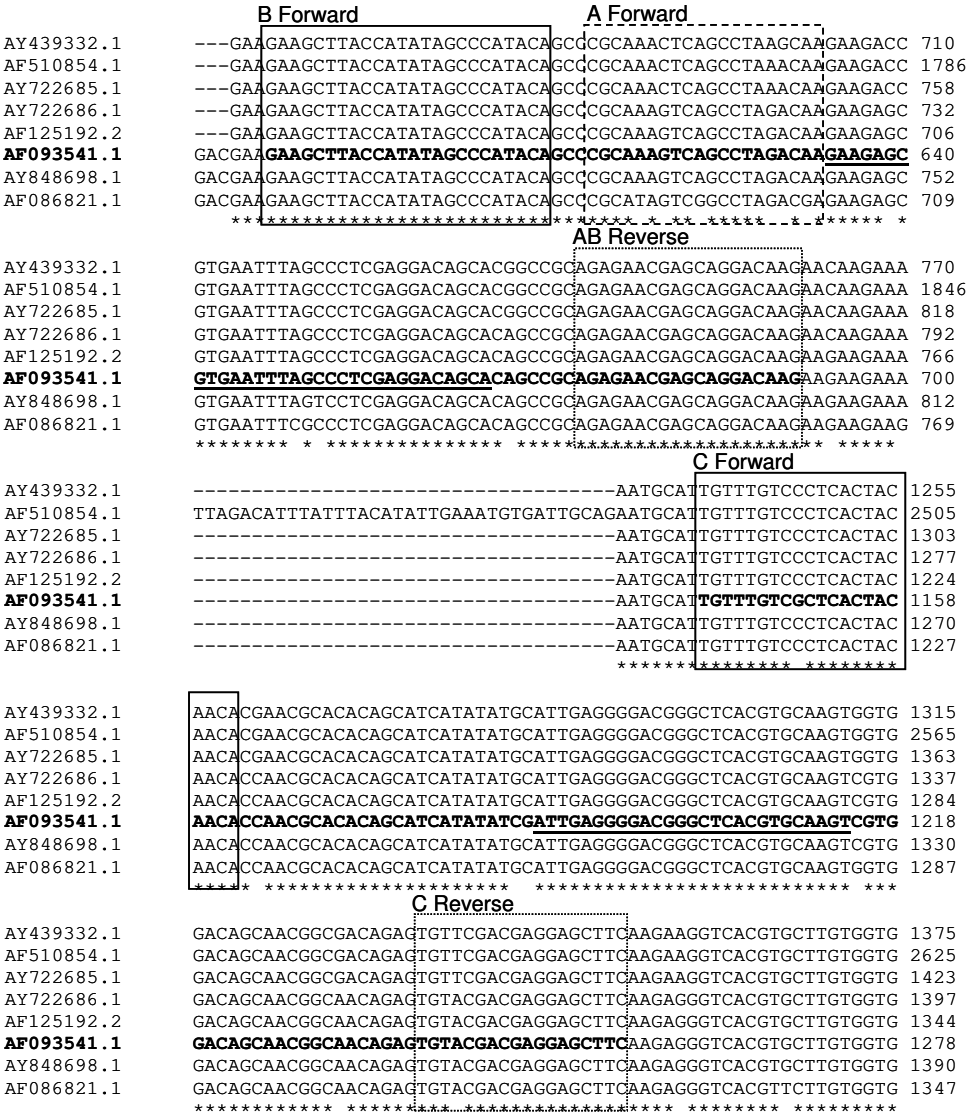


Figure 1: Alignment (ClustalW, http://www.ebi.ac.uk/clustalw/) of the most conserved region of 8 Ara h 3 gene sequences (asterisks indicate nucleotides identical in all sequences). In bold letters the amplicon of the primary Ara h 3 sequence (AF 093541). Primers (boxed) and probes (underlined) are indicated for PCR A, B, and C.

Three primer sets were therefore manually designed: PCR A (78 bp amplicon, nucleotides 614 - 691 of AF093541), PCR B (105 bp amplicon, nucleotides 587 - 691 of AF093541), PCR C (114 bp amplicon, nucleotides 1142 – 1255 of AF093541). The alignment shown in Figure 1 depicts the homology of the primer and probe sequences with 8 sequences of the Ara h 3 gene family. "B forward" and "AB reverse" primers are fully homologous with all 8 sequences, while "A forward" shows a perfect homology with 4 of the 8 sequences and contains 2 or 3 mismatches with the others. The "AB probe" is completely homologous with 3 sequences but is likely to be able to hybridize to the other 5 since it contains only a single mismatch. "C forward" has been synthesized with a single mismatch compared to the original Ara h 3 sequence (AF093541) to achieve a perfect homology with the other 7 sequences, while "C reverse" is homologous with 5 of them. "C probe" can perfectly hybridize all the gene family members considered.

Specificity test

As described above the specificity of the primer and probe sets was checked by homology searches to DNA sequences present in the NCBI data base. In addition to this we analysed the specificity experimentally by using DNA extracts from a variety of different foods. All the food materials listed in Table 1 were used for DNA extraction, the DNA was quantified and its potential for PCR amplification was assessed. The amplification of non-coding regions of chloroplast DNA served as positive control for all plant-derived foods. For non-plant derived matrices, the presence of PCR inhibitors was investigated by spiking the samples with a positive control. In all DNA extracts of the foods listed in Table 1 amplification of either the control (chloroplast) DNA or the spiked DNA was observed. Employing the Ara h 3 primer and probe sets (PCR A, B, C) resulted in a failure to amplify DNA in any of the foods listed in Table 1, except for peanut. We can therefore conclude that all three assays are specific for peanut detection.

Moreover, the target sequences can be amplified from all five different peanut varieties that are included in the IRMM-481 Peanut Test Material Kit (mean Ct values 22.3 ± 1 for the five varieties and considering all three assays).

All samples were analysed by means of gel electrophoresis and amplified DNA was only detected in peanut samples. The lengths of the amplification products, as determined by gel electrophoresis were in agreement with the size of the amplicons.

Table 1: list of food matrices tested in order to verify the specificity of PCR A, B and C. The positive or negative amplification of the DNA extracts was identical for all three assays. Positive (Pos) refers to results with Ct values and Negative (Neg) refers to results where the Ct values were undetermined

Food matrix:	PCR test	Food matrix:	PCR test
Barley (*Hordeum vulgare*)	Neg	Chicken (*Gallus gallus*)	Neg
Millet (*Panicum miliaceum*)	Neg	Cattle meat (*Bos taurus*)	Neg
Buckwheat (*Fagopyrum esculentum*)	Neg	Potato (*Solanum tuberosum*)	Neg
Oat (*Avena sativa*)	Neg	Sunflower seeds (*Helianthus annuus*)	Neg
Rye (*Secale cereale*)	Neg	Rape seeds (*Brassica napus*)	Neg
Spelt (*Triticum dicoccum*)	Neg	Olives (*Olea europaea*)	Neg
Rice (*Oryza sativa*)	Neg	Cocoa beans (*Theobroma cacao*)	Neg
Maize (*Zea mays*)	Neg	White Lupin (*Lupinus albus*)	Neg
Wheat (*Triticum aestivum*)	Neg	Sesame seeds (*Sesamum indicum*)	Neg
Green Pea (*Pisum sativum*)	Neg		
Lentils (*Lens esculenta*)	Neg		
Mung beans (*Phaseolus aureus*)	Neg	Peanut (*Arachis hypogaea*):	
Chick peas (*Cicer arietinum*)	Neg	Jumbo Runner from USA	Pos
Soya (*Glycine max*)	Neg	Virginia from USA	Pos
White beans (*Phaseolus vulgaris*)	Neg	Runner from Argentina	Pos
Almond(*Prunus dulcis*)	Neg	Virginia from China	Pos
Brazil nut (*Bertholletia excelsa*)	Neg	Common Natal from South Africa	Pos
Cashew nut (*Anacardium occidentale*)	Neg		
Hazelnut (*Corylus avellana*)	Neg		
Pecan nut (*Carya illinoensis*)	Neg		
Pistachio (*Pistacia vera*)	Neg		
Macadamia nut (*Macadamia spp*)	Neg		
Walnut (*Juglans regia*)	Neg		

PCR and DNA extraction optimization

For the optimization of the real-time PCR methods different parameters were analysed. This included the primer concentrations and the annealing and elongation temperatures. In addition to this, different PCR mastermixes were compared.

High specificity in primer annealing and low Ct values for the amplification were aimed at in the optimization study. The lowest Ct values were obtained by using a low annealing temperature (55 °C) but the specificity of primer annealing improves by increasing temperature, therefore we chose 60 °C for annealing temperature. Further optimization of the method showed that utilizing the Taq Polymerase from Applied Biosystems and an elongation temperature of 72 °C (instead of 60 °C, as suggested by the manufacturer), yielded the lowest Ct values.

Next, we aimed to optimize the DNA extraction. A high yield of extraction, a high purity and a good amplificability of the DNA are important requirements to facilitate the detection of the target

sequences and to obtain low Ct values. Therefore, an investigation was carried out to determine the optimum extraction method for the purification of DNA from peanuts.

DNA from raw and roasted peanut was extracted in duplicate with 15 different methods based on different extraction principles. Some were commercial kits that were utilized according to the manufacturers' instructions; others were traditional extraction methods, while the last group employed a traditional extraction buffer combined with a commercial DNA purification system. The yield of extraction was measured by quantifying each sample in a fluorimetric assay. Since peanut is known to contain a high level of fats and oils which can potentially be co-extracted with genomic DNA and inhibit the PCR, we verified the amplificability of the DNA by employing PCR amplification of non-coding regions of chloroplast DNA. Subsequently each DNA extract was amplified by all three PCR methods (A, B, and C) in replicates, and the mean of the Ct values was used to evaluate the performance of the extraction methods.

From the data shown in Table 2, it is clear that with most methods the yield of extracted DNA from raw peanut is higher than that from roasted peanut.

Regarding the amplification of peanut DNA with the three different methods many differences could be observed. In general there is no clear correlation between the amount of DNA extracted and the Ct value obtained as exemplified by the comparison of results obtained with commercial kit **i** and the traditional method **ix**. With the latter the amount of DNA extracted is 10 times higher (376.49 ng/µl) then that obtained with the commercial kit (30.34 ng/µl). But, since similar Ct values were obtained the DNA extract of method **ix** is most likely not pure which affects the PCR efficiency and results in a high standard deviation (± 2).

Clear differences in the amplification of raw and roasted peanut were observed. For the commercial kits **iii**, **iv** and **v**, the higher extraction yield of raw peanut is reflected in lower Ct values compared to roasted peanuts**.** In contrast to this, utilizing commercial kit **i** yields three to four times more DNA from raw compared to roasted peanuts but the amplification rate is quite similar (Ct values of 21-22). Kit **vi** extracts DNA with similar yields from raw and roasted peanut (5-9 ng/µl) but the efficiency of amplification is strongly decreased for the roasted peanut extract (from Ct around 28 to Ct around 36). Unfortunately after employing methods **i**, **ii**, **iii** and especially **vii** amplification of the extraction blank was observed and therefore those method are considered not to be reliable for our purpose.

The results obtained with the traditional extraction methods (**ix, x, xi**), indicate that raw and roasted peanuts are complex matrices that require special purification to remove PCR inhibitors. A traditional extraction buffer, such as the CTAB buffer, was therefore combined with purification on

columns, resulting in a lower Ct value (Ct around 20) and lower standard deviation (±0.2) than the traditional method without further purification (Ct from 20 to 22, ±2).

Method **xiii** and **xiv** are also based on a combination of a traditional buffer (containing guanidine hydrochloride) and a commercial purification system. Unfortunately the yield of extraction is very low (method **xiv,** 7 ng/µl) which leads to high Ct values (Ct around 23) or the standard deviation is too high (method **xiii**, ±2-3).

Among all the different extraction methods, the best combination of a traditional buffer and a further purification was found to be method **xv**: which yields a high DNA concentration, one of the lowest Ct values for both raw and roasted peanut (Ct value 18-20) while also a very low variability between the replicates was observed for all three PCRs.

These results led us to use the guanidine hydrochloride extraction buffer combined with the Wizard® DNA Clean up system (method **xv**) for all the DNA extractions from raw and roasted peanut and also from processed foods like cookies.

Table 2: DNA concentration (ng/µl) of the extracts from raw and roasted peanut as obtained with 15 different extraction methods. For each extraction method, the amount of starting material is mentioned in brackets. Replicates of extraction were analysed in duplo with all three PCR methods (A, B, C) utilizing a fixed volume of 5 µl of DNA extract. The Ct values reported refer to the mean value of the four replicates of amplification. The respective standard deviation is given. Ct values of the two blanks of extraction are listed for each PCR assay.

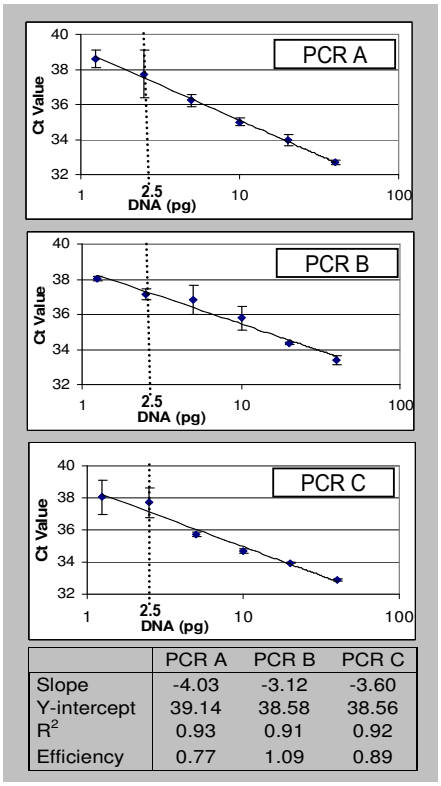
		DNA conc. (ng/uL)		Ct values and *standard deviation*								
		RAW	ROASTED	RAW peanut			ROASTED peanut			BLANK OF EXTRACTION		
		peanut	peanut	A	B	C	A	B	C	A	B	C
I	DNA extraction kit (2g)	30.34	9.84	22.02	21.76	21.56	21.94	22.19	22.01	38.33	-	-
	(GMO & Allergen; Tepnel)			*±0.24*	*±0.69*	*±0.43*	*±0.12*	*±0.12*	*±0.1*	-	-	-
II	DNAMITE Plant Kit extraction (500mg)	2.58	2.55	36.48	39.23	38.79	-	-	-	-	-	37.39
	(Microzone Limited)			*±0.97*	-	-	-	-	-	-	-	*0.61*
III	Wizard® Magnetic DNA Purification	69.06	3.73	19.84	19.68	19.63	25.32	25.61	25.04	38.37	38.12	-
	System for Food (200mg), (Promega Co.)			*±0.45*	*±0.37*	*±0.31*	*±0.09*	*±0.05*	*±0.03*	*±0.17*	-	-
IV	Wizard® Magnetic DNA Purification	25.57	5.40	21.78	22.32	21.93	25.50	26.29	25.24	-	-	-
	System for Food (1 g), (Promega Co.)			*±0.86*	*±0.79*	*±1.39*	*±0.92*	*±1.28*	*±1.24*	-	-	-
V	GenElute TM Plant Genomic DNA Kit (100mg)	17.00	2.53	23.07	22.97	22.29	29.12	29.48	29.18	-	-	-
	(Sigma)			*±0.23*	*±0.24*	*±0.04*	*±0.09*	*±0.15*	*±0.17*	-	-	-
VI	Easy-DNATM Kit for genomic)	9.29	5.35	26.89	28.06	28.80	34.12	36.67	36.62	-	-	-
	DNA isolation (100mg), (Invitrogen Ltd)			*±1.60*	*±2.44*	*±2.85*	*±0.53*	*±1.57*	-	-	-	-
VII	SureFood® PREP Allergen Kit (250mg)	49.47	40.27	-	-	-	24.72	23.03	25.59	36.32	35.40	36.31
	(R-Biopharm/Congen)			-	-	-	*±3.58*	*±0.33*	*±3.14*	*±0.61*	*±0.73*	*±1.43*
VIII	Nucleon PhytoPure plant and fungal DNA	113.20	x	-	-	-	x	x	x	-	-	-
	extraction kit (Amersham Biosciences Corp.) (1g)			-	-	-	x	x	x	-	-	-
IX	CTAB	376.49	x	21.18	22.37	20.24	x	x	x	-	-	-
	(100mg)			*±2.01*	*±2.8*	*±1.82*	x	x	x	-	-	-
X	S.M. Aljanabi et al. protocol	83.82	254.52	28.23	-	24.60	-	-	-	-	-	-
	(1g)			*±0.67*	-	*±0.32*	-	-	-	-	-	-
XI	S.M. Aljanabi et al. protocol modified	93.35	29.71	-	-	-	29.02	-	33.04	-	-	-
	(1g)			-	-	-	-	-	-	-	-	-
XII	CTAB+SureFood® PREP Allergen kit (1g)	155.62	x	20.57	20.37	20.30	x	x	x	-	-	-
	(R-Biopharm / Congen)			*±0.13*	*±0.22*	*±0.30*	x	x	x	-	-	-
XIII	Guan. Hydr.+DNeasy Plant Mini Kit (1g)	124.78	x	20.55	26.44	21.37	x	x	x	-	-	-
	(Qiagen)			*±1.75*	*±3.73*	*±1.88*	x	x	x	-	-	-
XIV	Guan.Hydr.+SureFood® PREP Allergen kit (1g)	7.64	x	23.51	23.17	22.22	x	x	x	-	-	-
	(R-Biopharm / Congen)			*±0.12*	*±0.03*	*±0.14*	x	x	x	-	-	-
XV	Guan.Hydr. Wizard® DNA Clean up system (2g)	199.52	41.63	18.92	18.67	17.98	20.15	20.29	19.98	-	-	-
	(Promega Co.)			*±0.10*	*±0.42*	*±0.17*	*±0.12*	*±0.17*	*±0.03*	-	-	-

Sensitivity

In order to protect the health of allergic consumers, methods need to be sensitive and capable of detecting trace amounts of peanut. Therefore the performance of all three PCR assays was analysed to determine how many copies of peanut genome equivalents they are able to detect. For this purpose we used a DNA mixture of equal aliquots of the 5 different peanut varieties included in the standard material IRMM-481. The extracted DNA mixture was diluted in water to establish two different standard curves: from 1 pg to 100 ng and from 1.25 pg to 40 pg. The amplification was carried out over 40 cycles, which is our defined cut-off, that avoids false positive signals that might appear due to artefacts of amplification when the thermal cycle is too long (Kolmodin et al., 2000). Figure 2 shows that PCR A, B and C are all capable of detecting 2.5 pg peanut DNA, while also aliquots of 1.25 pg peanut DNA can still be detected within 40 cycles of amplification, but the amplification curves show an increased variability of results around 1.25 pg, which renders the results not reliable enough for this sensitivity test. The peanut genome size is estimated to be around 2.8 Mb (Guo et al., 2004; Dhillon et al., 1980), and by applying the appropriate calculations (http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf), it turns out that the weight of the entire peanut genome is around 3 pg. All three real-time PCR assays can detect less than 3 pg, which implies that they can all detect less than 1 genome equivalent. This can be explained by the fact that the target sequences are located in the conserved area of the Ara h 3 gene family and therefore are repeated in the peanut genome. These results confirm our choice for Ara h 3 as target sequence. Previously Stephan and Vieths (Stephan and Vieths, 2004) have described an assay that can detect 10 copies of peanut genome, while the real-time PCR method developed by Hird et al. (Hird et al., 2003) was show to have a detection limit of 34 genome copies (Brežná et al., 2005). The conventional PCR designed by Watanabe et al. (Watanabe et al., 2006) which is also based on the principle of targeting a multicopy gene is capable of detecting less than one genome equivalent but only in qualitative format, as detected by gel electrophoresis.

Another parameter that can be derived from these standard curves is the PCR efficiency. The slope of the log-linear phase of the amplification curves is a reflection of the reaction efficiency. The efficiency can therefore be calculated by the following equation: Efficiency = $(10^{(-1/\text{slope})})-1$. In order to obtain accurate and reproducible results, reactions should have an efficiency as close to 100 % as possible and an ideal slope of -3.3. The PCR efficiency for all three PCR assays in the case of the high range dilutions (1 pg to 100 ng) was in the range of 91-93% with a slope ranging from -3.49 to -3.53. Moreover, as shown in Figure 2, the PCR efficiency remained high (in a range of 77 to 100%) when only the smaller amounts of DNA template (1.25 pg to 40 pg) were used for

amplification. This means that all three assays are sensitive and efficient enough to be applied for the detection of trace amounts of peanut.



	PCR A	PCR B	PCR C
Slope	-4.03	-3.12	-3.60
Y-intercept	39.14	38.58	38.56
R^2	0.93	0.91	0.92
Efficiency	0.77	1.09	0.89

Figure 2: Standard curves of PCR methods A, B and C obtained by amplifying 1.25, 2.5, 5, 10, 20 and 40 pg peanut DNA diluted in water. The table below lists the slope, the y-intercept and the R2 of each curve and the calculated PCR efficiency of each assay (calculated by using SDS 2.2, Applied Biosystems, UK).

Peanut detection in a model food

As described above for the determination of the specificity, the efficiency and the sensitivity of all three PCR assays, pure peanut DNA extracts were analysed. However, the real application of the methods lies in the detection of peanut traces in food products. Therefore real food samples, spiked with peanut were developed. Since the health of allergic consumers is most likely to be endangered by the accidental ingestion of peanut traces in complex food matrices that can contain a variety of different ingredients and might have undergone a heat treatment, we developed a processed food matrix for bakery products to which peanut was added before food processing. The peanut mix IRMM-481 was used for spiking. Cookies with different peanut contents (0, 1, 10, 100, 1000, 10000 mg kg^{-1}) were prepared in a pilot bakery plant according to a standard recipe. The resulting material was used to study the performance of the real-time PCR methods developed in this study.
All different cookies were extracted in duplicate, the DNA content of the extracts was quantified and replicates of amplification were performed. Since the yield of extraction was comparable for all the samples (ranging from 65 to 100 ng/µl), a fixed volume of DNA extract (5 µl) was utilized in the PCR analyses. Figure 3 shows an example of an amplification plot obtained with PCR B. The cut-off for amplification was set at 40 cycles, which is very low compared to other published

methods (Stephan and Vieths, 2004). Despite this restrictive setting, 10 mg kg^{-1} peanut can clearly be detected in the cookies by all three PCR methods and this level was considered to be the limit of detection for the PCR assays (Table 3). After PCR the samples were also analysed by means of gel electrophoresis and a single band of amplified DNA was detected which was in agreement with the size of the amplicons. This level of 10 mg kg^{-1} is within the range of detection limits that are relevant for the detection of potentially hazardous residues of undeclared allergens in foods (Hefle and Koppelman, 2006) but it is higher than the sensitivity that can be reached by some ELISA assays (Poms et al., 2005; Yeung and Collins, 2005), although these data are not directly comparable to our results due to differences in the preparation of the spiked food products. The sensitivity of ELISA assays is often assessed by using food matrices like chocolate or cookies spiked with pure peanut protein extract where the peanut does not undergo the heat treatment typical for industrial production. The material we used for our experiments aimed to better represent products from the market. The linear response of the PCR amplification ranges from 10 mg kg^{-1} to 10000 mg kg-1 indicating that all three PCR methods are capable of a quantitative analysis of the peanut content in this complex, processed food matrix.

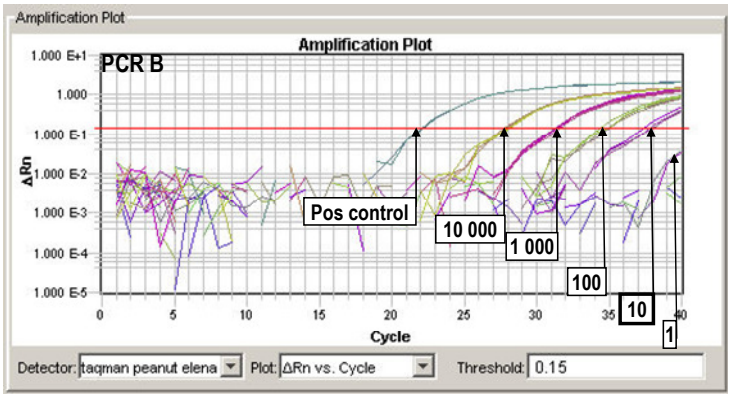


Figure 3: Amplification plot of PCR B (105 bp), obtained by analysing the DNA extracted from cookies containing 10000, 1000, 100, 10 and 1 mg kg-1 of peanut, and a positive control (pure peanut DNA).

Table 3: Mean Ct values of 4 replicates and their standard deviations as obtained from the analysis of DNA extracted from cookies containing 10 000, 1000, 100, 10, 1 mg kg-1 peanut and amplified with PCR methods A, B and C.

	PCR A	PCR B	PCR C
Peanut (mg kg^{-1})	Ct value ± std dev	Ct value ± std dev	Ct value ± std dev
Blank	-	-	-
1	-	-	39.26[a] ± -
10	37.40 ± 0.79	37.76 ± 0.36 [b]	37.78 ± 1.25 [b]
100	34.33 ± 0.28	34.74 ± 0.23	34.24 ± 0.28
1000	31.30 ± 0.11	31.39 ± 0.13	30.99 ± 0.11
10 000	27.60 ± 0.06	27.92 ± 0.09	27.18 ± 0.06

[a] In 3 out of 4 reactions the Ct values were undetermined
[b] In 1 out of 4 reactions the Ct value was undetermined

Limit of Detection (LOD) and Limit of Quantification (LOQ) are parameters indicative of the method performance for quantitative analyses. In quantitative PCR, LOD and LOQ need to be experimentally determined because the blank values normally used for the calculation (LOD is generally defined as the signal strength of a blank increased by three times the standard deviation while LOQ is the signal strength of a blank increased by six-ten times the standard deviation of the blank) is defined as *undetermined* (truncated at zero).

In practice, the two sensitivity values obtained by our methods describe the linear range within which peanut DNA in a water solution (until less than 1 copy of genome equivalent) or in a processed food matrix (from 10 to 10000 mg kg^{-1}) can be quantified with suitable accuracy as determined in a method validation (Range of Quantification, ROQ) (Lipp et al., 2005).

The ROQ established by employing a food matrix might differ from that employing pure peanut DNA, since food matrices can contain polysaccharides, proteins, phenolic compounds, and proteins that might be cross-linked to DNA through carbohydrates. All such compounds that can be present in complex food matrices and might be co-extracted with the target DNA could affect the efficiency of the PCR reaction. Such "PCR inhibitors" may vary in their extent to which they affect individual PCR analyses. This effect of the model matrix was determined by comparing the amplification plot obtained by analysing subsequent dilutions of peanut DNA (IRMM-481) in water or in blank cookie extract. The calibration curves obtained were found to be very similar. Table 4 shows the comparison of the slopes, y intercepts and R squares values. The data indicate that the PCR reaction is not affected by any extracted cookie matrix components. Studies on the matrix effect on PCR detection of peanut are generally not reported despite the fact that such assays have been applied to several different food products (Stephan and Vieths, 2004; Hird et al., 2003; Watanabe et al., 2006).

In order to assess the performance of the real-time PCR methods developed in this study we employed two commercial test kits for the detection of peanut, one based on real-time PCR and the other one on conventional PCR. Unfortunately the use of the real-time PCR kit combined with the extraction kit produced by the same supplier yielded false positive results after analysis of the blank of extraction. Moreover, despite the declared limits of detection in food products of 10 mg kg^{-1} for the real-time PCR kit and "low level" for the conventional PCR based kit, we were unable to reliably detect less than 50 mg kg^{-1} peanut in cookies. This emphasises the good reliability and sensitivity of the methods developed in this study.

Table 4: The slope, y-intercept, and R square derived from the three curves (PCR A, B, C) obtained by amplifying peanut DNA (IRMM-481) diluted in water or in blank cookie extract.

	PCR A		PCR B		PCR C	
	Blank cookies	Water	Blank cookies	Water	Blank cookies	Water
Slope	-2.91	-2.85	-3.00	-2.95	-2.87	-2.92
Y-Intercept	27.05	26.63	27.26	26.91	26.67	26.39
R^2	0.99	0.98	0.99	0.98	0.99	0.97

Conclusions

The development of specific and sensitive analytical methods for the quantification of hidden allergens in food products is a great challenge. Over recent years several DNA based assays for allergenic foods like peanut (Stephan and Vieths, 2004; Hird et al., 2003; Watanabe et al., 2006; Sforza et al., 2005; Rossi et al., 2006), wheat (Köppel et al., 1998) , olive (Aché et al., 2002), apple (Son et al., 1999), hazelnut (Holzhaueser et al., 2000, 2002; Herman et al., 2003; Arlorio et al., 2007), cashew nut (Brzezinski, 2006), pea (Brežná et al., 2006a), and walnut (Brežná et al., 2006b) have been developed and serve as good alternatives to protein-based detection methods.

In this study we developed three different real-time PCR assays targeting conserved DNA sequences in the Ara h 3 gene family. All three PCR assays are specific for peanut, have a detection limit of less than 1 copy of genome equivalent, while in a complex processed food matrix 10 mg kg^{-1} peanut can clearly be detected, furthermore no influences of the cookie matrix were observed. The cookie material used represents a DNA-rich food matrix which is likely to negatively impact the sensitivity that can be achieved. Despite this, the developed methodology is capable of detecting 10 mg kg^{-1} peanut in cookies which is low enough to be comparable to the range of threshold doses eliciting allergic reactions in sensitized people (Krska et al., 2004). The linear range within which peanut DNA can be quantified with an appropriate accuracy is between 10 and 10 000 mg kg^{-1} in cookies. This represents a major advantage compared to the very limited working range of ELISA test kits. The fact that the real-time PCR methods described here, in contrast to ELISA analyses, do not require several dilutions of samples to quantify a high levels of peanut implies that the method is efficient in terms of labour and costs.

Hird et al. (Hird et al., 2003) reported a real-time PCR assay and presented data related to dilutions of pure peanut DNA extract and several real food samples, while Stephan and Vieths (Stephan and Vieths, 2004) have shown a comparison between samples analysed by ELISA and a real-time PCR method. Watanabe et al. (Watanabe et al., 2006) have reported a conventional PCR method combined with gel electrophoresis. In all those cases PCR was only used as a qualitative method for peanut detection in food (for real-time PCR results Ct values are given, but peanut content was not

determined). For the two-real-time PCR methods, no strict settings on the number of PCR cycles or on the standard deviation between the replicates were defined. Nevertheless, Stephan and Vieths (2004) reported that Ct values higher than 45 with standard deviation of ± 2 are likely to identify positive samples, but for such samples a correlation with the ELISA rates could no longer be observed. In the present study the use of a model food matrix with a precise peanut content, enables us to apply the real-time PCR technique for a quantitative determination of the peanut content. We conclude that the three methods developed in this study can be applied in the near future to detect peanut traces in commercial food products.

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Chapter 3

The effect of heat treatment on the detection of peanut allergens as determined by ELISA and real-time PCR

Peanut allergic reactions that can result from the ingestion of even very small quantities of peanut represent a severe threat to the health of sensitised individuals. The detection of peanut traces in food products is therefore of prime importance. Peanut traces which can be (unintentionally) present in a multitude of food products have usually undergone one or more food processing steps like roasting and baking. Therefore, the methods designed to detect such traces have to be capable of detecting heat treated peanut. Commonly used methodologies designed to detect peanut traces in food products are enzyme-linked immunosorbent assays (ELISAs) that detect peanut specific proteins, and polymerase chain reaction (PCR) based methods targeting peanut specific DNA. A comparative analysis of such methods was performed and the impact of heat treatment on peanut kernels as well as the impact on a peanut-containing food matrix was investigated. Our results show that heat treatments have a detrimental effect on the detection of peanut with either type of method and that both types of methods are affected in a similar manner.

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Introduction

Food allergy is a serious public health problem. Around 1–2 % of the population suffer from some type of food allergy and even higher prevalence levels (up to 8 %) are estimated for children (Sicherer et al., 2003, 2004; Helm et al., 2000; Ortolani et al., 2001). The severity of the reactions triggered by food allergens vary from mild urticaria to potentially lethal anaphylactic shocks, even after the ingestion of minimal doses of the allergenic food. It is estimated that in the United States about 30,000 anaphylactic reactions to food are treated in emergency departments and that food allergy results in 150-200 deaths each year (Sampson, 2003). Peanuts and tree nuts are responsible for the majority of deaths attributed to food allergies (Bock et al., 2001; Pumphrey, 2001).

Unfortunately, avoidance is the only means allergic consumers have to protect their health. Therefore, EU legislation has been issued aimed at informing the consumer about the content of food products by means of a manditory labelling of allergenic food ingredients. The new EU Directive 2007/68/EC, which is an amendment to Directive 2000/13/EC (European Parliament and Council, 2000, 2007), provides a list of 14 groups of allergenic foods that manufacturers are required to declare on the labels if any of them are used as ingredients in pre-packed foods, regardless of their quantity. Reliable analytical methods for the detection of hidden allergens in foodstuff are therefore required by the food industry and food control authorities to guarantee food safety for allergic consumers.

Food allergens, the components capable of triggering allergic reactions, are usually proteins. The structure and chemical properties of proteins are known to be influenced by food processing techniques such as boiling, baking, roasting, curing, and other types of food processing.

It has been reported that the allergenic activity of the peanut allergens Ara h 1, Ara h 2, and Ara h 3 increases with roasting while it decreases with frying or boiling (Maleki et al., 2000; Beyer et al., 2001). This decreased allergenicity was suggested to be a contributing factor in the lower prevalence of peanut allergy in China where peanuts are mainly boiled or fried compared with the United States where they are consumed roasted (Beyer et al., 2001).

Thermal processing of foods is known to result in a degradation of food constituents, or in their modification. Such modifications can result from Maillard reactions, which are important in the development of flavour and colour in peanuts (Maleki et al., 2000; Chung and Champagne, 2001). In addition, interactions with other constituents of the food matrix may occur as a result of thermal processing.

All these modifications often result in a decreased protein solubility which affects protein extraction from food matrices. In peanut it has been shown that protein solubility decreases due to industrially

applied processing techniques like roasting (Chaissagne et al., 2007; Mondoulet et al., 2005; Poms et al., 2004). Furthermore, the integrity of the target proteins could be changed during food processing leading to a further reduction in their detectability, since any analytical method based on the detection of markers for allergenic ingredients depends on an efficient extraction of such markers and their structural integrity.

Currently, Enzyme-Linked Immunosorbent Assay (ELISA) is probably the method that is most commonly used by the food industry and official food control agencies to assess the presence of allergens in food products. ELISAs are immunochemical based tests, which can be either designed to specifically recognize one allergenic protein, or to detect a pool of proteins contained in the protein extract of the allergenic food (Krska et al., 2004). They usually employ IgG antibodies raised in animals, which bind allergen specific epitopes. But, in case of modifications of the protein structure or matrix effects this antibody binding could be affected.

An alternative to ELISA is provided by DNA based methods. Several of such methods have recently been developed for the detection of peanut traces in food products (Hird et al., 2003; Stephan and Vieths, 2004; Sforza et al., 2005; Rossi et al., 2006; Watanabe et al., 2006; Scaravelli et al., *in press*). The target molecules of these detection methods are not proteins but specific DNA sequences which are amplified by the Polymerase Chain Reaction (PCR). This technique is commonly used in the detection of genetically modified organisms (GMO) and, when applied to allergen detection, it represents an indirect investigation of the allergenic ingredients. The target sequences can be designed on the gene coding for the allergenic protein or simply, on a species-specific region in the genome of the allergenic food. For peanut allergen detection, several real-time PCR assays have been developed (Hird et al., 2003; Stephan and Vieths, 2004; Scaravelli et al., *in press*) that, like ELISA, have the potential to be applied for quantitative purposes. Although DNA based methods target a different analyte compared to immunological methods like ELISA, food processing also affects the integrity and extractability of this target.

Consumers are almost solely exposed to heat treated peanuts since, for direct consumption, peanuts are usually roasted. Moreover, peanuts are widely used in different preparations like snacks and biscuits, and in the confectionary industry. This implies that they undergo additional (thermal) food processes. It is therefore of utmost importance to investigate the negative effects that food processes like thermal treatments exert on the detection of peanut specific markers being either proteins or DNA.

Previously several studies have reported a qualitative comparison of ELISA kits and DNA methods for the analysis of processed food products (Stephan and Vieths, 2004; Watanabe et al., 2006; Piknova et al., 2007), but such studies have not addressed the influence of food processing on either

type of detection methodology. In this study three real-time PCR assays all targeting DNA sequences coding for Ara h 3 (Scaravelli et al., *in press*) are used in parallel with two different ELISA kits for the analysis of raw and roasted peanut as well as on a heat treated model food matrix containing peanut. The main focus of this study lies on the effect that heat treatments on peanut kernels and on cookies containing peanut have on the detection of peanut protein and DNA targets. The reactivity of the different detection methods towards processed allergenic foods, to which sensitized consumers are exposed to, was investigated. This allows an assessment of the possibility to confirm analytical results for the detection of peanut traces in processed food products obtained by ELISA with real-time PCR assays or vice versa.

Materials and Methods

Food samples

Five varieties of peanut, identical to the 5 peanut components included in the IRMM-481 peanut test material (IRMM, Belgium), were analysed. Each component corresponds to a single peanut variety that underwent a specific heat treatment, being blanching or roasting as listed in Table 1. The peanuts were obtained from IMKO (The Netherlands) and Duyvis (The Netherlands). In addition to this, raw peanuts were obtained from the same suppliers. Except for peanut component **d**, for which only blanched peanut was available (blanching conditions: 20 minutes at 90ºC), while the raw peanut component **c** was provided by Golden Peanuts (USA). In addition to the single varieties the mixture of the 5 processed materials (component **f** of IRMM-481) was analysed.

A fine peanut powder of the mixture of the 5 peanut materials (component **f** of IRMM-481) had been used to spike cookies as described previously (Scaravelli et al., *in press*). The peanut content in the cookies was approximately 10 000, 1000, 100 and 10 mg kg^{-1}. All cookies were baked at 180 °C with a standard baking time of 16 minutes, or for different periods of time being 11, 21 or 26 minutes. All spiked cookies were ground, sieved on 0.7 µm sieves and aliquots of 15 or 30 g of the resulting powder were packed under vacuum in aluminium foil bags and stored at -20 °C until use.

DNA extraction and quantification

DNA extraction was performed combining a guanidine hydrochloride based extraction and the Wizard® DNA Clean up system (Promega, Madison, USA) as described previously (Scaravelli et al., *in press*). Samples of 2 g (± 0.1 g) pure peanut and 5 g (± 0.1 g) cookies were used for DNA extraction Each sample was extracted in duplicate and the purified DNA from peanut and cookies was eluted in 100 µl and 130 µl TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) respectively.
DNA quantification was carried out as described previously (Scaravelli et al., *in press*).

Real-time PCR

PCR A, PCR B and PCR C all targeting Ara h 3 (Scaravelli et al., *in press*) have been applied.
For sample analysis two separate DNA extractions were performed while two aliquots of 5 µl DNA extract were amplified in two separate reactions. In the case of the analysis of pure peanut extracts, DNA was diluted 1:10 and 5 µl of solution was added to each reaction. The dilution was taken into account for the expression of results.
For all analyses the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, UK) was used with an automatic baseline setting and a threshold value of 0.15.
Each 96-well plate included three non-template controls (NTCs) and two positive control samples. For each sample the final Ct values used for the calculation of quantitative results refer to the mean value of four replicates (two replicates of extraction and two replicates of amplification). For quantification purposes a cut-off Ct value of 40 was set, but higher Ct values up till 45 were considered acceptable for qualitative results.

ELISA methods

Two commercially available test kits targeting a specific peanut allergen were used: Biokits Peanut Assay kit (Tepnel BioSystems, UK), targeting Ara h 1; Peanut Residue ELISA kit (ELISA Systems, Australia), targeting Ara h 2. The ELISA test kits were used according to the manufacturer's instructions, by following the enclosed protocols. For sample analysis two separate protein extractions were performed and analysed in two separate reactions.

Methods of analysis

Homogeneity test

The homogeneity of the cookie material was tested by employing the BioSystems peanut allergen test kit as well as the three PCR methods. Cookies of the two lowest spiking levels, containing either 10 or 100 mg peanut per kg cookie, that were baked for 16 minutes (standard baking time) were analysed for this purpose. Two samples from ten different bags of each concentration level were randomly taken and analysed in triplicate. The averages of the optical density (OD) or Ct values were subjected to a statistical analysis of variance (ANOVA), in order to assess the homogeneity of the cookie material.

Calibration model

A statistical approach was followed to analyse all data obtained with the real-time PCR for quantitative purposes. To identify the best calibration model Microsoft Excel was used and the guidelines of Miller and Miller were followed (Miller and Miller, 2005). The equations obtained were confirmed by Statgraphics Plus (version5.1; StatPoint.Inc, Virginia).

Calibration model with DNA dilutions (real-time PCR A, B, C)

DNA extracted from IRMM-481 component **f** (a mixture of 5 peanut materials) was used to prepare subsequent DNA dilutions in water to establish a standard curve from 1 pg to 100 ng (1 pg, 10 pg, 100 pg, 1 ng, 10 ng, 100 ng) for each PCR method. Three separate reactions were performed for each concentration level on two different days, using different stocks of PCR reagents. The equation of the calibration curve was generated by applying a weighted linear regression and plotting the Ct values against the logarithm of the corresponding DNA quantity (calibration points). The weighting factors were calculated according to the following equation (Miller and Miller, 2005):

$$w_i = s_i^{-2} / \sum_i (s_i^{-2} /n)$$

where w_i : individual weights of each calibration points

s_i : standard deviation of the replicates per each calibration point

n : number of calibration points

The curves obtained for each of the three PCR assays were used to interpolate unknown samples and to determine the amount of peanut DNA in the respective PCR reactions.

For the analysis of unknown samples, two DNA extracts were obtained from each material and two aliquots of both DNA extracts were taken for the analysis with each of the PCR assays. The average

of those 4 values was interpolated in the respective PCR standard curve to define the peanut DNA content of the unknown samples.

Calibration model with cookies containing peanut (real-time PCR C)

A weighted linear regression was also applied to the Ct values obtained by amplifying DNA extracted from peanut containing cookies that were baked for 16 minutes (standard baking time), using PCR C. For each of the four calibration points (peanut spiking levels of 10, 100, 1000, 10000 mg kg^{-1}), four PCR reactions were performed (two replicates per each of the two DNA extracts). The PCR reaction was then repeated twice on different days using different stocks of PCR reagents. The final set of analysis was composed of twelve PCR reactions for each calibration point. The curve was generated by plotting the Ct values against the logarithm of the corresponding peanut content. The weighting factors were calculated according to Miller and Miller (Miller and Miller, 2005) and the equation was confirmed by Statgraphics Plus.

For the lowest and highest calibration points (10 and 10000 mg kg^{-1}), the confidence interval was calculated. Each 96-well plate analysed included a control of the calibration curve by the inclusion of two independent PCR reactions per DNA extract of the 10 and 10000 mg kg^{-1} calibration points. When the averages of the Ct values of both calibration points were within the confidence intervals previously defined by the calibration curve, the analysis results of the plate were considered reliable. The calibration curves were updated every time the DNA of the calibration points ran out or when new extraction buffer stocks were used.

When the calibration curve was used to interpolated the Ct values obtained by the analysis of peanut containing cookies baked for different periods of time, the variability of the method in predicting a concentration value from a certain measured Ct value (mean of four replicates) was calculated as the "*prediction limits*" with 95 % confidence level, using Statgraphics Plus.

Calibration model with ELISA kits

The manual of the Biokits Peanut Assay kit suggests using a linear regression for quantitative purposes but, since the R-squares obtained were always around 0.91, polynomial regression was applied in order to obtain a better fit (R-square 0.99). The peanut content (mg kg^{-1}) of the six calibration points (0, 1, 2, 5, 10, 20 mg kg^{-1}) was plotted against the mean (three replicates) of their respective ODs.

The manual of the Peanut Residue ELISA kit does not specify the use of any regression for quantification but only suggests comparing the ODs obtained from the unknown samples with the standards provided. In order to predict the peanut content of unknown samples, also in this case,

polynomial regression was applied. The curve was based on six calibration points (0, 0.125, 0.25, 0.5, 1 and 2.5 mg kg^{-1}). The standards provided in the kit were 0, 1, 2.5 and 5 mg kg^{-1} but since the 5 mg kg^{-1} standard always returned saturated OD values, three lower concentrations were introduced by means of dilution to increase the data points for a calibration curve. The lower concentrations all returned OD values that were above the OD of the blank + 3 times the standard deviation.

Regarding the analysis of unknown samples, two protein extracts were obtained from each sample material tested, and both protein extracts were analysed in duplicate. The concentration of peanut material in the sample was determined by interpolating the corresponding average of the four ODs with the calibration curve obtained with the standards provided in the kit.

Repeatability test

Ten samples from each concentration level of the cookie material (16 minutes baking time) were randomly selected. Three independent measurements per sample were performed with the BioSystems peanut allergen test kit and with PCR C. The average of each set of three measurements was considered representative of the sample analysed. The relative standard deviations (or coefficients of variation) were calculated on the averages of the OD values for the ELISA method and on the Ct values for the real-time PCR method according to this formula:

$$CV\ (\%) = ((s_i) / x_i) * 100$$

Where:

s_i= standard deviation between the averages of the sets of three measurements

x_i= average of all the measurements

Results and discussion

Peanut detection by real-time PCR and ELISA

Prior to its consumption, peanut usually undergoes heat treatments like blanching or roasting. The IRMM-481 peanut test material, which was used in this study, includes five peanut varieties from different geographical origins exposed to five different industrial heat treatments (Table1). Therefore it represents a material that might be comparable to the peanuts the consumer is exposed to. Each variety (component **a**, **b**, **c**, **d**, **e**) and the mixture of the five peanuts (component **f**) were analysed separately using the two ELISA kits targeting Ara h 1 and Ara h 2 and the three real-time

PCR assays as quantitative methods. Component **f** of IRMM-481 that contains a mixture of different peanut varieties exposed to different heating processes was chosen as the best material for the establishment of calibration curves for the PCR assays. Therefore, dilutions of the DNA extracted from this material were employed to generate calibration curves for the three real-time PCR assays as described in the Material and Methods section. For the data analysis and the generation of calibration curves we followed the guidelines of Burns et al (Burns et al., 2004). Due to the heteroscedastic distribution of the data points, linear weighted regression was applied to describe the calibration models, moreover as suggested by Burns et al., individual Ct values collected for each calibration point, instead of their respective averages, were used to calculate the equation of the curves. The calibration curves obtained by the three PCR assays were characterized by R^2 values close to 1 (PCR A 0.998, PCR B 0.997, PCR C 0.997), and as determined from their individual slopes the efficiency of the PCR reactions ranged from 95 to 97%. The curves were generated by plotting Ct values against the logarithm of the amount of peanut DNA, aimed at the interpolation of unknown samples.

The ELISA test kits that were used were supplied with their own standards that were utilised to establish calibration curves for the interpolation of unknown samples.

To perform a comparative analysis with real-time PCR and ELISA, both sampling and the number of measurements performed were equal for both methodologies. However, different calibrants were utilized for either ELISA or PCR, and the final quantification of each peanut sample was differently expressed (in mg peanut per kg for ELISA, or ng peanut DNA for PCR). In order to evaluate and compare the data obtained from both detection methods, all results were normalized to the mixture component **f** of IRMM-481.

Table 1: List of the five peanut materials as included in the IRMM-481 test material.

Variety	Origin	Heat treatment
(a) Runner	Argentina	dry hot air roasting, 140ºC, 20min
(b) Comon Natal/Spanish	South Africa	dry hot air roasting, 140ºC, 13 min
(c) Virginia	USA	oil roasting, 145 ºC, 25 min
(d) Virginia	China	oil roasting, 140 ºC, 9 min
(e) Jumbo Runner	USA	Blanching, 100ºC, 50 min

Effect of heating on peanut detection

In order to study the effect of the industrial processing as applied to the different components of IRMM-481 we compared the analysis of its components with their raw counterparts by means of ELISA and real-time PCR. In addition, the analysis of the five different raw peanut varieties

allowed us to investigate the variability in the allergen detection as caused by differences in peanut variety.

The left part of Figure 1 shows the results for the raw peanuts of all 5 varieties as determined by ELISA, while the left part of Figure 2 shows the real-time PCR results for the same material. A clear variation in analytical response was apparent for both methodologies. Results obtained with the ELISA kit targeting Ara h 1 varied from 109 to 300 % of the normalized value of component **f** of IRMM-481, while using the ELISA kit targeting Ara h 2 the variability was slightly lower ranging from 136 to 274%. Thus, the ELISA analysis of the different varieties can show a 2- to 3-fold difference in signal.

On the contrary, analysis of the same material with the three real-time PCR methods showed a much reduced variability with signals differing by a factor 1.4 for PCR A, 1.7 for PCR B and 1.5 for PCR C. Those observations suggest that the concentration of the two proteins targeted by the ELISA kits varies considerably amongst the different varieties tested. In contrast to such variable protein levels the DNA concentration is stable in cells, which supports our observation that the targeted peanut DNA represents a more stable marker.

Interestingly, the three PCR methods showed a similar behaviour with all of them detecting the highest signals for raw E followed by raw A, raw B or raw C and finally raw D. For ELISA clear differences were observed with the highest Ara h 1 signal measured in raw E and the lowest in raw A while the highest Ara h 2 signal was measured in raw C and the lowest in raw B. Similar variabilities were reported previously in (validation) studies of ELISA kits targeting different proteins (Whitaker et al., 2005; Koch et al., 2003).

The right part of both Figures 1 and 2 shows the results obtained after analysis of the different industrially processed peanut varieties. A comparison with the left part of the figures visualises the effect of heat treatment, and it is apparent that the roasting processes dramatically reduces peanut detection when either ELISA kits or PCR methods are employed.

By comparing the ELISA results obtained with the mixture IRMM-481, which is composed of blanched and roasted peanuts, the estimation of peanut content in raw kernels can be double or three times higher than the value of the mixture. This is consistent with other studies that have compared raw and roasted peanuts analysed by ELISA (Koch et al., 2003; Chassaigne et al., 2007) and seems to be largely caused by a difference in protein extraction efficiency (Poms et al., 2004; Kopper et al., 2005; Chassaigne et al., 2007). Poms et al. and Chaissagne et al. observed that in roasted peanut the yield of extraction using commercial buffers can drop to 20 % of the level of raw peanut, because of reduction in protein solubility (Poms et al., 2004; Chaissagne et al., 2007).

Looking at individual peanut varieties, Ara h 1 is more sensitive to heat treatment than Ara h 2. The signal of three out of five processed varieties falls below 50 % of the value of the mixture for Ara h 1 (samples A, B and C); while in the case of Ara h 2 this happens only once (sample B). In addition to this, component **e**, that only underwent blanching, did not show a decrease, but a modest increase of Ara h 2 compared to its raw counterpart. A light heat treatment like blanching can probably induce cell breakage which facilitates protein extraction.

Figure 2 shows that the roasting process also resulted in a decrease of detection when DNA was the target. Even though DNA, as a macromolecule, exhibits a relatively high chemical stability, there is a broad spectrum of chemical and enzymatic reactions that cause DNA modification and/or degradation. Thermal treatments, are known to, contribute to such reactions (Bauer et al., 2003, 2004; Moreano et al., 2005; Engel et al., 2006). Generally the drop in peanut DNA detection caused by processing is more evident than with protein detection by ELISA (Figure 1). The PCR signals obtained after analysis of raw peanuts were 2 to 5 fold above those of the processed (standard) mix, while for ELISA detection this difference ranged only from 1.1 to 3 fold.

When the results of the individual processed varieties were compared, a lower variability was observed after PCR analysis compared to analysis with ELISA. This is apparent from the observation that after real-time PCR analysis only one processed variety (B) showed a more than 50 % deviation from the standard, while after Ara h 2 detection 2 processed varieties (B and E) and after Ara h 1 detection even 4 (A, B, C and E) out of the 5 varieties showed a more than 50 % deviation from the standard.

Having assessed that the signal obtained after analysis of raw peanuts is clearly higher than that of the respective roasted peanuts when real-time PCR is employed, we investigated the integrity of the DNA molecules as extracted from raw and processed peanuts. Raw, blanched (100 °C, 30 minutes), mild roasted (140 °C, 12 minutes) and strong roasted (140 °C, 20 minutes) peanuts from a single variety were analysed. Quantification of the DNA extracts from raw (177 ± 4 ng/µl), blanched (194 ± 3 ng/µl), mild roasted (76 ± 8 ng/µl) and strong roasted (48 ± 11 ng/µl) peanut indicated that the DNA extraction yield considerably decreases with increasing heat treatment. Therefore lower amounts of template (extracted peanut DNA) are added to the PCR reactions of roasted samples since normally a fixed volume of DNA extract is used.

In addition to the yield we investigated the quality or integrity of the extracted DNA by studying its amplificability. For this purpose the DNA content was standardized before amplification by PCR. The amplification of 1 ng template DNA in each PCR reaction resulted in similar Ct values (28.4 ± 0.4) for all samples regardless of the heat treatment (Figure 3). Therefore we conclude that a major limiting factor in DNA detection as well as in protein detection is the extractability of peanut DNA

or protein. This study also showed that DNA obtained from strong roasted peanuts was perfectly amplifiable by all three PCR assays with a comparable efficiency even at low concentrations, which shows that the extracted DNA is of good quality.

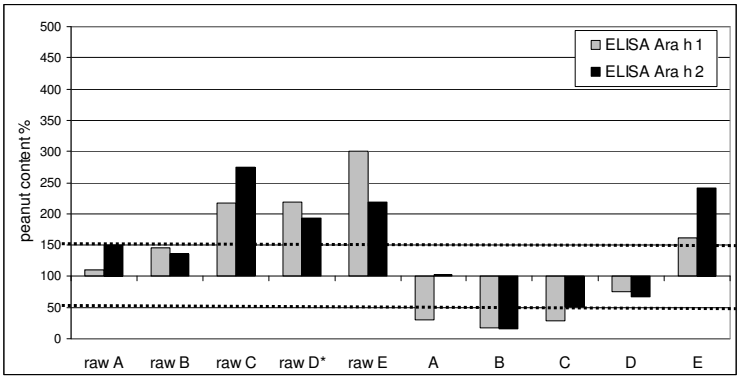

Figure 1: Estimation of the peanut content in different raw and roasted peanut varieties (listed in Table 2) as determined with an ELISA kit targeting Ara h 1 or with an ELISA kit targeting Ara h 2. All values are normalized to the peanut content determined in the mixture (IRMM-481 component f).
* This peanut material was blanched under mild conditions.

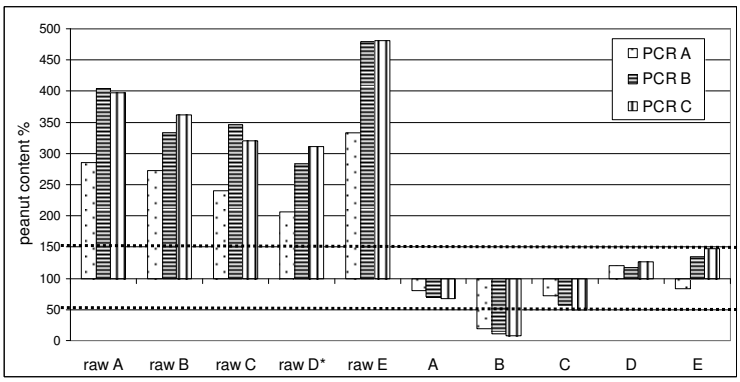

Figure 2: Estimation of the peanut content in different raw and roasted peanut varieties (listed in Table 2) as determined with the real-time PCR A, B or C methods. All values are normalized to the peanut content determined in the mixture (IRMM-481 component f).
* This peanut material was blanched under mild conditions.

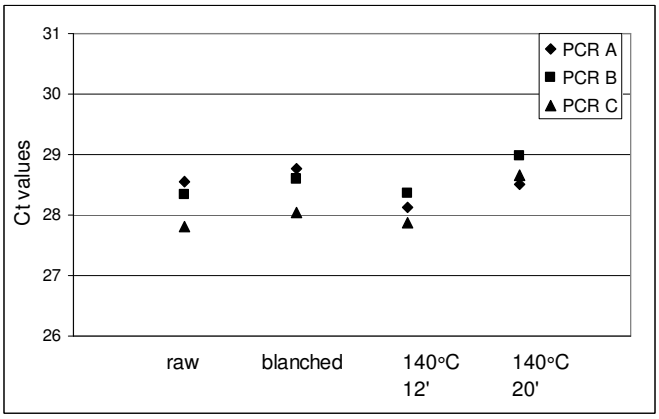

Figure 3: Comparison of the amplification of DNA of raw, blanched (100 °C, 30 minutes), mild (140 °C, 12 minutes) and strong (140 °C, 20 minutes) roasted peanut (Runner) with the three real-time PCR assays (A, B, C). In each PCR reaction an aliquot of 1 ng of DNA was used for amplification.

ELISA and real-time PCR analysis of a model food matrix

Accidental ingestion of peanut by an allergic consumer most likely occurs when peanut is hidden in a food matrix. Such peanut-containing foods can contain a variety of different ingredients and might have undergone a heat treatment. Therefore we studied the detection of peanut in real food samples and compared DNA and protein based detection methods. For this purpose, we employed a

processed food matrix (cookies), representative for bakery products, to which peanut was added before food processing (baking), as described previously (Scaravelli et al., *in press*).

All different cookies were analysed in duplicate with all three PCR assays and the two ELISA kits. The peanut content in the cookie material was quantified with the ELISA kits as described in the Material and Methods section, whereby quantification was based on the protein standards provided in the kits. However, such standards were not available for quantification by means of the real-time PCR methods. For the proper comparison of ELISA and real-time PCR detection all results were normalized to the mixture component **f** of IRMM-481. Previously we have shown that analysis of this cookie material with the three PCR assays gave a linear response within a concentration range of 10 mg kg^{-1} to 10000 mg kg^{-1} for cookies baked 16 minutes (Scaravelli et al., *in press*), therefore this material was used to establish calibration curves.

Due to its method of preparation the ground cookie material was likely to be homogeneous. This presumed homogeneity was tested by utilizing the ELISA kit targeting Ara h 1 as well as the three PCR methods. For this purpose cookies containing peanut at the two lowest levels (10 and 100 mg kg^{-1}) were analysed. Statistical analysis of variance (ANOVA) was applied to the values obtained with three PCR assays and the ELISA kit. This revealed that the F-value for both concentration levels was below the critical value of 3.02 ($p = 0.05$) as determined with either method, which implies that, with a 95 % probability, the cookie material can be considered homogeneous regardless whether DNA or protein was detected. PCR and ELISA methods gave similar results, which supports the applicability of the three real-time PCR assays for homogeneity testing.

Having assessed that the material was homogeneous, it was used for the establishment of a calibration curve for the real-time PCR C. The curve obtained, plotting Ct values against the logarithm of the concentration ($y = -3.275 \log(x) + 41.067$; PCR efficiency: 102 %) was used to interpolate samples with an unknown peanut content and to estimate the peanut content in cookie material that underwent different periods of baking.

In contrast to PCR analysis, ELISA calibration curves are characterised by a very limited working range. This implies several dilutions of sample extracts from material with a high peanut content which likely affects the precision of the method. We therefore tested the repeatability of the different methods. For this purpose PCR C and the ELISA kit targeting Ara h 1 were utilized.

The repeatability of analytical methods like real-time PCR is an important parameter to consider as stated by the European Network of GMO laboratories (ENGL, 2005). It is defined as the standard deviation of test results obtained under repeatability conditions. In this study, the coefficient of variation (CV) was determined for both the real-time PCR and ELISA methods. Figure 4 shows a comparison of the calculated CVs and shows that a decrease in peanut content leads to an increase

in measurement variability for the real-time PCR method. This confirms our observation that the real-time PCR calibration curves were characterised by a heteroscedastic distribution of data points, whereby the largest standard deviation corresponded to the lowest peanut level. The calculated CVs for the ELISA method show a similar trend although here the cookie material containing 10 mg kg^{-1} of peanut returned a CV value that was lower than expected.

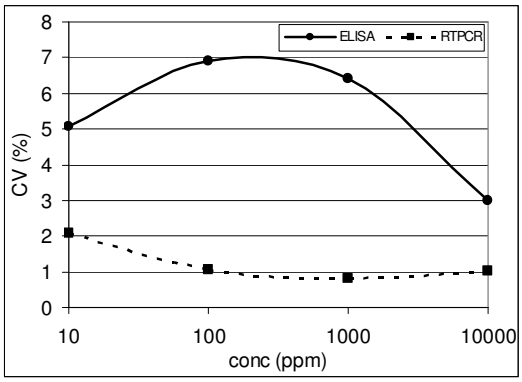

Figure 4: Coefficient of variation (CV %) calculated from the Ct values obtained with real-time PCR C or from the ODs obtained with the ELISA kit targeting Ara h 1. Results are based on the analysis of 10 cookie extracts per concentration level (standard baking time of 16 minutes).

Despite the fact that the CV values generally follow the same trend for both methods (an increase at lower concentrations), for ELISA the CV values ranged from 3 to 5 % while real-time PCR showed a higher repeatability with CV values ranging from 1 to 2 %. The latter CV values fall within the range of CV values reported by Piknova et al. (Piknova et al., 2007) (1 % < CV < 3 %) who analysed subsequent dilutions of DNA by real-time PCR to detect hazelnut allergens.

The effect of heat treatment in a model food matrix

Food products that contain peanut as an ingredient, or in which peanut is unintentionally present (e.g. as a result of contamination during the production process) constitute a clear threat to the health of allergic consumers. Such food products often contain peanuts that have undergone two subsequent heat treatments, as exemplified by roasted peanuts used as ingredients for baked cookies. This is most dangerous for sensitized consumer since it can trigger allergic reactions while such a product might not be identified as peanut-containing (not listed on the label) and analytical detection of peanut fails due to the manufacturing processes applied.

Up till now, only limited data are available about DNA or protein stability during processing in complex food matrices. In the case of DNA, most of the available studies are focussed on the detection of DNA sequences of genetically modified organisms in food and mainly focus on the degradation of the genomic DNA up to a certain amplifiable length (Moreano et al., 2005; Chen et

al., 2005; Murray et al., 2007). Other studies highlight the effect of different parameters like pH, time and temperature on the extent of DNA degradation. Bauer et al identified the heat treatment and acidic condition as the main parameters responsible for DNA degradation (Bauer et al., 2003, 2004).

In this study we investigated the effect exerted by heat treatment of a complex food matrix on the detection of peanut. Cookies with different peanut contents that were baked for either 11, 16, 21 or 26 minutes were analysed by using PCR C and the two ELISA kits.

Figure 5 shows a comparison of the relative peanut contents measured over the range of baking times that were investigated. For the determination of the peanut content, the calibration curve based on the same cookie material baked for 16 minutes was applied. As expected, the detection of both the protein and DNA targets decreases with increased baking time. The standard material (baked at 180°C for 16 minutes) showed a comparable quantification with all three methods, close to the true value of 100 mg kg^{-1}. Prolonging the baking time for ten minutes resulted in a decrease of the estimated peanut content by a factor 10. Detection of the PCR target did not differ from detection of the Ara h 1 protein target, although the PCR detection was characterised by smaller prediction intervals. The ELISA kit targeting Ara h 2 generally followed the same trend, but returned higher estimations of peanut content after prolonged baking. This is in accordance with studies reporting a high heat stability of Ara h 2, and a better extraction efficiency of Ara h 2 from heat roasted peanuts compared to Ara h 1 (Chassaigne et al., 2007; Maleki and Hurlburt, 2004).

The Ct values of the analysis of the cookies that underwent different times of heat exposure were

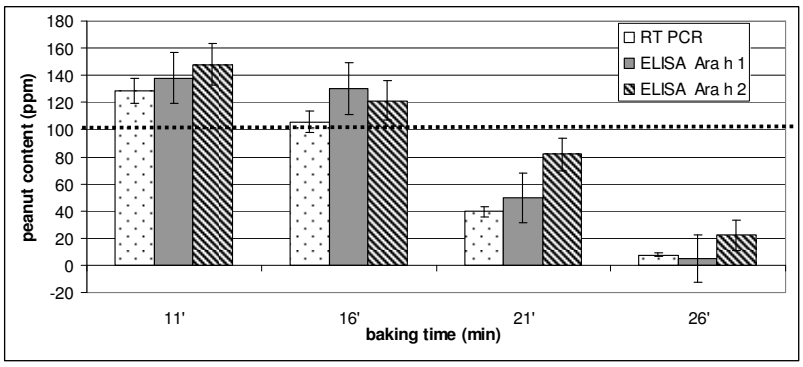

Figure 5: Estimation of the peanut content (ppm = mg kg-1) in cookies containing peanut at a level of 100 mg kg-1 and baked for 11, 16, 21 or 26 minutes. The measurements were performed with real-time PCR C and two ELISA kits (targeting Ara h 1 and Ara h 2). The error bars refer to the *prediction intervals* of each predicted value derived from the calibration curves

	10ppm		
	PCR A	**PCR B**	**PCR C**
11'	4 / 4	4 / 4	4 / 4
16'	4 / 4	4 / 4	4 / 4
21'	3 / 4 *	2 / 4 Φ	4/ 4 Φ
26'	3/ 4*	2 / 4	1 / 4

* 1 Ct value above 40

Φ 2 Ct values above 40

Table 2: The effect of baking time on the detection of peanut by means of the three PCR assays (A, B, C). The cookie material analysed contained peanut at a level of 10 mg kg-1 and was baked for 11, 16, 21 or 26 minutes. The results are presented as an index based on the number of positive results obtained after analysis of four samples.

investigated to study the sensitivity of the three PCR methods with regard to the heat treatment and to establish a Limit of Detection (LOD). Traditionally the LOD is often derived from the mean of blanks and the standard deviation of the blank, which can not be applied to quantitative PCR techniques as recently stated (Burns and Valdivia, *in press*), since negative controls do not return a zero measurand result or a specific value, instead they are just defined as "undetermined". An important parameter that needs to be fixed to define the LOD is the Ct cut off value. It is important that the Ct cut off value is not set too low in order not to omit true positive results but also not too high to include false positive results (Burns and Valdivia, *in press*). In this study two Ct cut off values were set, all Ct values below 40 were considered reliable for quantification purposes, and Ct values up till 45 were considered for positive qualitative results. The range between Ct values of 40 to 45 was used to define an experimental LOD relying on the ability of the real-time PCR method to discriminate a fluorescent signal from a sample in terms of the number of PCR reactions that returned results within this range of Ct values. Although the Ct values and therefore the estimated peanut content decreased with higher baking time all cookie material containing peanut at a level of 100 mg kg^{-1} or higher tested positive (data not shown). However, prolonging the baking time caused a drop in detection and resulted in several negative tests when the cookies containing peanut at a level of 10 mg kg^{-1} were analysed. Table 2 illustrates the results for this cookie material as analysed by the three PCR assays. Four separate reactions were carried out per method per baking time. The results show that at this low peanut content the prolonged heat treatment can prevent the detection of peanut traces in the cookies.

This represents a danger to erroneous interpretations. In the model matrix analysed here all analyses of the blank matrix samples returned without exception "undetermined" Ct values. Therefore, any positive test result cannot be assigned to the amplification of non-target DNA. Instead the occurrence of positive as well as negative test results for the same sample points at the fact that low levels of target analyte are giving rise to stochastic effects of sample heterogeneity resulting in erroneous interpretation. The fact that material with prolonged baking times returned Ct values above and below 40 (but less than 45), and is characterised by a lack of repeatability may indicate that the DNA was degraded by heat treatment, or that the extraction of peanut (target) DNA is reduced after prolonged baking. Both explanations implicate that only a few template sequences were available for the amplification. Degradation of DNA after thermal processing is reported by Hiramoto et al (Hiramoto et al., 1994) who have shown that DNA breakage takes place during food processing which is caused by the Maillard reaction of glucose and amino acids in an aqueous system. However, our results on the effects of heat treatments on the quality and quantity of the DNA extracted from whole peanut (as reported above) suggest that the decreased detection in the

material that had undergone prolonged baking most likely results from a decreased efficiency of extraction of the target DNA.

A different approach to define the limit of detection was applied to ELISAs. The ELISA kit targeting Ara h 1 sets its LOD at two times the value of the blank. Applying this LOD, all cookie samples containing peanut at a level of 10 mg kg^{-1} returned positive results, even at the higher baking times. The ELISA kit targeting Ara h 2 sets its LOD at a value equal to that of the chosen positive control sample. Since the positive control sample with the lowest peanut content is that of 1 mg kg^{-1}, this value was used as the LOD in our experiments. Applying this LOD, all cookie samples containing peanut at a level of 10 mg kg^{-1} returned positive results when the baking time was 11 minutes. However, after 16 minutes of baking only one out of four samples returned a positive result. On first instance this might be unexpected since Ara h 2 shows higher heat stability than Ara h 1 but, within peanut Ara h 1 is more abundant compared to Ara h 2 (Koppelman et al., 2001) which might explain our observations. Furthermore, our results are in line with those of an inter-laboratory validation study that included the same two ELISA kits as used in our current study. In the ELISA validation study a higher number of false positive results were obtained with the Ara h 2 ELISA, compared to the Ara h 1 ELISA kit after analysis of cookies containing peanut at a level of 2 mg kg^{-1} (Poms et al., 2005).

Conclusions

A major concern in the protection of allergic consumers is the availability of detection methods able to recognize the presence of trace amounts of allergenic ingredients in food products. Since information on clinical thresholds is very limited, there is a general agreement, that such detection methods should have a detection limit between 1 and 100 mg kg^{-1}(Poms and Aklam, 2004; Poms et al., 2003). A review (Poms and Anklam, 2004) on this topic lists the currently available methods, some of which are designed for a qualitative response only, while others are (semi-)quantitative methods reported to be capable of achieving a good sensitivity with LOD values within the mentioned range. Since rather diverse methodologies are employed for food allergen detection it is of importance to compare their performance. Recently several studies have embarked upon this issue and compared PCR and ELISA technologies designed to detect peanut traces in food products (Stephan and Vieths, 2004; Watanabe et al., 2006; Piknova et al., *in press*). Those studies have reported qualitative comparisons, and have largely focussed on the analysis of market samples. The lack of reference materials for allergen detection has brought individual research groups to develop

their own test material and their own experimental design, compromising a direct comparison of such studies.

In this study we compared two different ELISA kits, one targeting the allergenic protein Ara h 1 and the other Ara h 2, with three real-time PCR methods. The peanut test material IRMM-481 was utilised for calibration as well as for spiking. This study shows that, despite the completely different nature of the target molecules, protein or DNA, the performance of the different detection methods was very similar. A comparison of 5 different peanut varieties confirmed that the level of DNA is more stable than that of the proteins Ara h 1 or Ara h 2. As expected, roasting was found to strongly affect the detection of both target molecules, and our study shows that this decrease in detection follows a similar trend for both technologies. Despite this, we observed some variability which was higher between the two ELISA kits, compared to the variability between the PCR methods. The observed variability amongst ELISA kits is consistent with other studies (Whitaker et al., 2005) and is based on the fact that the ELISAs target different proteins and therefore resulted in different estimations of peanut content, which is not the case for the three PCR methods.

In addition a model food matrix representative of bakery products has been utilized and both ELISA as well as real-time PCR methods could be employed to confirm the homogeneity of this test material. This material was used to estimate the peanut content in cookies baked for different periods of time, to determine to which extent peanut DNA or protein detection is affected to the heat treatment in a complex food matrix. The two ELISA kits as well as the real-time PCR methods showed a similar trend, since prolongation of the baking time (26 minutes compared to the standard 16 minutes) resulted in a 5 to 10 times decreases in detection.

Overall, we can conclude from this study that the use of the three real-time PCR methods returns analytical results that are comparable to those obtained with the commercial ELISA kits that were tested. With regard to the effects of roasting of peanuts, or baking in a cookie matrix it was interesting to note that one of the ELISA kits showed a behaviour that was more similar to the real-time PCR method than to that of the other ELISA kit.

Both ELISA as well as PCR based methods have disadvantages. The first might return false positive results due to cross-reactivities of the antibodies, while the latter detects DNA and therefore does not investigate the presence of allergenic proteins. Therefore it is very important to be able to confirm a positive result obtained with one method with a different method. This study shows that ELISA kits and real-time PCR complement each other and that the one can indeed be used to confirm results obtained by the other.

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Chapter 4

Peanut allergen detection in chocolate and in products from the market by means of ELISA and real-time PCR

Peanut allergy is an increasingly important public health problem that has a strong impact on the quality of life of allergic consumers and their families. Since consumption of peanut can provoke allergic symptoms even at minimal doses, while higher doses can be life threatening, sensitized individuals need to avoid peanut containing food products. Such an avoidance strategy which is the only practical means to prevent allergic reaction from occurring is complicated by the fact that peanut is widely used in the confectionary industry and can accidentally end up in pre-packed food products. Different methods have been developed for peanut detection in foodstuffs either based on the detection of specific DNA sequences or proteins as target molecules. The broad range of food matrices in which peanut is utilized, stresses the need for methods capable of analysing a wide variety of matrices. For this reason, a previously developed real-time PCR method was optimized and extended to include next to cookies also chocolate matrices, thereby covering an important branch of the confectionary industry. A comparative study between the real-time PCR method and ELISA was performed on hundreds of market samples. Our results show that, although different target molecules are detected, real-time PCR and ELISA show a good agreement for the qualitative detection of peanut in real food samples. Quantitative analysis showed that factors like matrix composition, and especially cocoa content, influence the analytical results and might differently affect the performances of the two methods.

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Introduction

Peanuts contain high levels of protein and oil and are widely used as ingredients in food and confectionery products or in the production of oils and butter. Despite their popularity, the widespread use of peanuts by the food industry represents a threat to the health of peanut allergic individuals and is a limiting factor in their quality of life and that of their families (Sicherer et al., 2001; Avery et al., 2003). Peanut is known to contain a variety of proteins which includes eight allergenic proteins (Ara h 1 to Ara h 8) (Burks et al., 1998; Koppelman et al., 2001; Bannon et al., 2000; Koppelman et al., 2003; Scurlock and Burks, 2004; Mittag et al., 2004). The consumption of peanut by sensitised individuals is potentially life threatening (Sampson, 2003) and even at minimal doses it can trigger allergic reactions (Flinterman et al., 2006). Moreover, in the USA and in Europe recent studies on the prevalence of food allergy amongst children confirmed that peanut allergy is increasing in recent years with the latest estimates of the prevalence rates for children in the region of 1 % (Sicherer et al., 2003; Grundy et al., 2002; Hourihane et al., 2007). Furthermore, peanut is responsible for most of the reported deaths attributed to food allergies over the last 5-7 years (Bock et al., 2007; Pumphrey and Gowland, 2007). Since there is no cure or treatment for this disease, avoidance of the offending foods is at present the only practical means at hand to prevent allergic reactions to occur. The need to carefully adhere to such an avoidance strategy explains why food allergy inevitably results in social restrictions and has an impact on the quality of life.

Unfortunately it is well known that food allergic consumers occasionally experience allergic reactions from accidental ingestion of the offending food. Modes of accidental ingestion of peanut include eating at home, at school (for children), in a restaurant or by consuming food products containing hidden or undeclared peanut residues (Sicherer et al., 1998; Yu et al., 2006). In case of packaged foods, comprehensive labelling of allergenic ingredients is a useful tool for sensitive individuals and allows for the selection of appropriate foodstuffs based on the information on the label.

New legislation specifically addressed to the protection of allergic consumers has recently been introduced both in the USA and the European Union (EU). A new labelling law, the Food Allergen Labelling & Consumer Protection Act (FALCPA), came into effect in the USA at the start of 2006 (FALCPA, 2004) and requires the identification on the food label of ingredients derived from eight commonly allergenic sources. Whereas within the EU Directive 2000/13/EC, as amended by Directives 2003/89/EC and 2007/68/EC (European Parliament and Council, 2000, 2003, 2007), requires a mandatory declaration of 14 allergenic foods. Peanut being a major allergenic food is in both cases included within the legislation.

In addition to the declaration of ingredients, manufacturers and retailers sometimes voluntarily add information on the possible unintentional presence of allergenic ingredients found in pre-packed food (eg. "may contain peanut" or "this product is made in a factory that also produces peanut-containing products"). The use of such warnings is based on the fact that during the production process allergenic ingredients can easily and accidentally end up in finished foodstuffs. Such hidden allergens can occur in packaged foods for a variety of reasons, including ingredients-statements omission or errors, cross contamination by the use of shared equipment or facilities, errors by ingredient suppliers or manufacturing firm employees, undeclared use of rework or leftovers, the presence of ingredients derived from the allergenic source (Vierk et al., 2002; Hefle et al., 2007). According to recent analytical surveys, the majority of packaged food products with precautionary labelling statements contain no detectable peanut residues. Nevertheless, a substantial part of them do contain peanut (Pele et al., 2007) and levels of up to 4000 ppm have been found in food products bearing a precautionary warning (Hefle et al., 2007). The frequent use of precautionary warnings and the fact that (allergic) individuals are often not heeding the advice of precautionary warnings are dangerous developments that pose clear health risks (Hefle et al., 2007; van Hengel, 2007).

In order to change the attitude of sensitive individuals towards food labels, offering them consistent information about the content of foodstuffs is of prime importance. To support this, the development of reliable analytical methods capable of detecting the presence of trace amounts of food allergens are required as they are useful tools for the food industry and official food control agencies.

Nuts and peanuts are important ingredients in the chocolate and confectionary industry because of their technological characteristics; particularly thanks to their flavour, especially if they are roasted, they combine well with chocolate (Minifie, 1989). In a recent study 569 cookies and chocolate samples collected on the European market were analysed in order to detect hazelnut and peanut traces by utilizing ELISA test kits and to compare the analytical results with the information provided on the label (Pele et al., 2007). The robustness of several ELISA test kits designed to detect peanut traces in food products has been investigated in recent validation studies (Whitaker et al., 2005; Park et al., 2005; Poms et al., 2005). But, method development as well validation studies are performed with a limited number of model matrices that are often spiked in a manner that does not mimic incurred samples where the allergen has been incorporated into the sample during processing. When ELISA test kits are used for the analysis of a large number of market samples the complexity and variability of food matrices is enormous. This increases the chance that matrix components affect the detection of allergen traces. Theoretically, false positive results can be obtained when methods designed to target markers of allergenic ingredients also respond positively

to other components of food matrices (van Hengel, 2007). It is therefore prudent to utilize more than one method to confirm positive analytical results. The detection of peanut protein traces by ELISA might be confirmed with the use of a method based on the detection of peanut DNA. Recently several PCR-based methods have been developed (Stephan and Vieths, 2004; Hird et al., 2003; Watanabe et al., 2006), while the performance of three real-time PCR methods was shown to be comparable to ELISA (*Chapter 3*). Moreover, the effect of a heat treatment like baking was shown to have a comparable impact on detection by ELISA and the three real-time PCR methods (*Chapter 3*). In order to confirm analytical results obtained by ELISA after investigating food samples in a market survey (Pele et al., 2007) the real-time PCR method had to be extended for the detection of peanut traces in chocolate.

In this study we present an optimised DNA extraction method and an extension of the real-time PCR methods which was established with the use of a model chocolate matrix. This matrix is clearly of interest because it has been reported that consumption of chocolate confectionary increased between 1991 and 1996 in the majority of the countries analysed (in Europe and the USA) (Wells, 1999). Because of this increased consumption, chocolate confectionary might represent one of the most hazardous food categories for peanut allergic consumers, since chocolate and chocolate based products often include nuts or peanuts. The availability of the real-time PCR methods capable of detecting peanut traces in both cookie and chocolate samples allowed us to confirm the analysis of the market samples that had previously tested positive by means of ELISA.

Materials and Methods

Food samples

Chocolate preparation

Seven types of nut-free chocolate were kindly provided by Barry-Callebaut (Belgium). Information on their composition is listed in Table 1. All chocolates were ground with an Ultra-turrax (IKA, dispersing tool 25N), in liquid nitrogen.

A finely ground peanut powder consisting of a mixture of the 5 peanut components included in the IRMM-481 peanut test material (IRMM-481 component **f**; IRMM, Geel, Belgium) were used for spiking. Aliquots of 0.05 g peanut powder were added to 4.95 g of each different chocolate powder and then analyzed in order to test the effect of the cocoa content on peanut DNA detection.

A model milk chocolate matrix based on chocolate **G** (Table 1; 28 % cocoa content) was prepared containing peanut at 4 different levels (10, 100, 1000, 10000 mg kg^{-1}). For this purpose 970 g fine powder of milk chocolate **G** was mixed with 20.0 g cocoa butter (Barry-Callebaut, Belgium) containing 10 g peanut powder (test material IRMM-481; component **f**). First, the fine chocolate powder was pre-warmed in a warm water bath at 80 °C until the chocolate melted, while mixing with a spoon. The cocoa butter was also pre-warmed in a water bath at the same temperature and peanut powder was added. By gently mixing with a spatula, the cocoa butter / peanut mixture was mixed with the molten chocolate. After 15 minutes mixing in the water bath at 80°C, the chocolate mixture was transferred to a form and cooled to room temperature (no tempering was applied). The solid chocolate with a 10000 mg kg^{-1} peanut content that was obtained in this way was finely ground with an Ultra-turrax (IKA, dispersing tool 25N), in liquid nitrogen and dried at room temperature. Subsequent dilutions were prepared with the nuts-free chocolate powder ("blank") to obtain chocolates with lower peanut levels. An aliquot of 450 g blank chocolate was added to 50 g chocolate with a 10000 mg kg^{-1} peanut content and mixed with the Ultra-turrax (IKA, dispersing tool 25N) under liquid nitrogen for 5 minutes. The 1000 mg kg^{-1} chocolate obtained was then used to prepare the 10 fold lower concentration level by addition of blank chocolate powder. The same was done up to a final concentration of 10 mg kg^{-1} of peanut in milk chocolate. All spiked chocolates were packed under vacuum in aluminium foil bags, and stored at -20 °C until use.

Cookie and chocolate samples

A total of 103 different types of cookies and 105 different types of chocolates purchased for a previously reported market survey (Pele et al., 2007) were selected for analysis. Those food products had been obtained from shops and supermarkets in 10 European countries (Austria, Belgium, Bulgaria, Czech Republic, Germany, Hungary, Poland, Romania, Slovakia and The Netherlands) and analysed with ELISA kit and dipstick. The food samples selected for real-time PCR analysis included all samples that had tested positive for peanut by ELISA analysis, and or by dipstick analysis. In addition to this all samples in which peanut was declared in the list of ingredients. Furthermore, 15 cookie and 12 chocolate samples that had tested negative with both ELISA and dipstick were selected.

DNA analysis

DNA extraction for cookie samples

DNA from cookies was extracted as described previously in *Chapter 3.*

DNA extraction for chocolate samples

The DNA extraction method for cookie samples was optimized to be applicable to chocolate samples. Two extraction additives, non-fat skimmed milk powder (cat. No. 54650, Biomol, Hamburg, Germany) and fish gelatine (cat. No. G7765, Sigma-Aldrich, St Louis, MO, USA), were used in combination with the guanidine hydrochloride based extraction buffer and the Wizard® DNA Clean up system (Promega, Madison, USA) for DNA purification (Scaravelli et al., *in press*).

Different aliquots (0.5, 1.0, 1.5, 2.5 g) of milk powder were added to 25 ml falcon tubes containing 5 g (± 0.1 g) chocolate powder containing 10000 mg kg^{-1} peanut. Aliquots of 10 ml extraction buffer (10 mM TRIS, 150 mM NaCl, 2 mM EDTA, 1% SDS, pH 8) and 2.5 ml guanidine-HCl 5M were then added to the tubes. Alternatively gelatine was used as additive, which was added directly to the extraction buffer. Samples of 5 g (± 0.1 g) chocolate powder containing 10000 mg kg^{-1} peanut were weighted into a 25 ml falcon tube and 10 ml pre-warmed (50°C) gelatine-containing extraction buffer (10 mM TRIS, 150 mM NaCl, 2 mM EDTA, 1% SDS, 1x Gelatine pH 8) and 2.5 ml guanidine-HCl 5M were added to the slurry.

From this step on, the protocol that was followed was largely based on the one described previously (*Chapter 3*). After incubation of the samples at 50 °C overnight, lipids were removed by extraction with 10 ml chloroform; the mixture was thoroughly mixed and followed by centrifugation at 9000 rpm for 15 minutes. The supernatant was collected in 2 ml eppendorf tubes and 16 µl Proteinase K (10 mg/ml) was added to each tube. The samples were incubated at 55 °C for 30 minutes, after which they were centrifuged at 14000 rpm for 15 minutes. The supernatant was transferred into a new 2 ml eppendorf tube, and 5 µL RNAse (100 mg/ml) was added. RNA digestion was carried out at 40 °C for 20 minutes. The solution was then transferred into a 14 ml falcon tube and of 2 ml DNA-binding resin (Wizard® Plus Minipreps DNA purification system) was added which was mixed thoroughly by inverting several times. The resin was loaded onto a minicolumn according to the manufacturer's instructions and subsequently washed with 3 ml 80 % isopropanol. Each sample was eluted from the column with 130 µl pre-warmed (70 °C) TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0).

The DNA extraction method for chocolate matrices that resulted in the better DNA yield and a better amplification was then employed to extract the DNA from all the samples from the market. This method used gelatine as extraction additive and all DNA extracts were diluted 10 or 20 fold.

Real-time PCR

PCR C designed on the target sequences of Ara h 3 genes as described previously (Scaravelli et al., *in press*) has been applied for the analysis of the milk chocolate model matrix. For this, two samples

were extracted per concentration level and two PCR reactions were performed per DNA extract. In addition, one sample of each DNA extract was spiked with pure peanut DNA extract in order to check for PCR inhibition.

For the analysis of the market samples, one DNA extraction was performed for each sample and four aliquots of 5 µl DNA extract were amplified in four separate reactions. One of the PCR reactions of each sample was spiked with pure peanut DNA extract in order to check for PCR inhibition. In case PCR inhibition was observed the DNA extract from the cookie sample was diluted 1:20 in water and the PCR analysis was repeated. For all chocolate samples, each DNA extract was diluted 1:10 and 1:20 in water and analysed in four PCR reactions; one of which was spiked with pure peanut DNA extract.

For all analyses the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, UK) was used with an automatic baseline setting and a threshold value of 0.15.

Each 96-well plate included three non template controls (NTCs) and two positive control samples. For each sample the final Ct values used for calculation of quantitative results refer to the mean value of the three replicates of amplification.

Protein analysis

ELISA methods

The commercially available Biokits peanut assay kit (Tepnel Biosystems, Deeside, UK), a sandwich-type ELISA, targeting the allergenic protein Ara h 1, was used according to the manufacturer's instructions

Methods of analysis

Homogeneity test

The homogeneity of the chocolate material was tested by employing the BioSystems peanut allergen test kit and the PCR C real-time PCR method (*Chapter 3*). The two lowest concentrations (10 and 100 mg kg^{-1}) of the spiked milk chocolate were analysed with real-time PCR C and all concentration levels were analysed with the ELISA kit. Two samples from ten different bags of each concentration level were randomly taken and analysed in triplicate. The averages of the optical density (OD) or Ct values were subjected to a statistical analysis of variance (ANOVA), in order to assess the homogeneity of the chocolate material.

Calibration model

In order to analyse all data obtained with real-time PCR for quantitative purposes a statistical approach was needed as described previously (*Chapter 3*). Calibration models for cookie and chocolate model matrices were generated by using Statgraphics Plus (version 5.1; StatPoint.Inc, Virginia, USA). This was used to determine the curves characteristics like slope, intercept, R^2 adjusted for the degree of freedom) and the respective PCR efficiency in the different matrices.

Calibration model for cookies matrix

A weighted linear regression was applied to the Ct values obtained by amplifying DNA from cookies containing peanut (10, 100, 1000, 10000 mg kg^{-1}) using PCR C as previously described (*Chapter 3*).

Calibration model for chocolate matrix

Four concentration levels of peanut spiked milk chocolate (10, 100, 1000 and 10000 mg kg^{-1}) were used to generate a calibration model as described previously (*Chapter 3*). The PCR reaction was repeated over a period of six days while using different stock solutions of PCR reagents. The final set of analysis was composed of 24 PCR reactions per calibration point. Because of the different level of PCR inhibition in milk chocolate compared to dark chocolate, DNA extracts were diluted 1:10 and 1:20 in water and two calibration models were generated. The calibration model based on a 1:10 dilution was mainly used to interpolate milk chocolate samples, and the calibration model based on a 1:20 dilution was employed to interpolate dark chocolate samples.

Repeatability test

The repeatability test was carried out as described previously for cookies (*Chapter 3*). Ten samples from each concentration level of the model chocolate matrix were analyzed with the BioSystems peanut allergen test kit (Tepnel) and with PCR C. For real-time PCR analysis DNA extracts were diluted 1:10 in water. The signals collected (Optical Densities or Ct values) were used to calculate the relative standard deviation.

Results and discussion

DNA extraction method optimization and cocoa content effect

Chocolate and chocolate based products represent a category of very difficult matrices for DNA analysis because of their high polyhenol and polysaccharids content that can reduce yield and purity of the extracted DNA (Gryson et al., 2004; Gryson et al., 2007). Polyphenols in their oxidized form can prevent extraction of protein or DNA by a covalent binding of these molecules whereas polysaccharides can form a viscous material which inhibits the activity of the Taq Polymerase (Porebski et al., 1997; Aljanabi et al., 1999; Khanuja et al., 1999; Katterman and Shattuck, 1993). In addition to this, several studies warn about the possible presence of other inhibitors of the PCR reaction like carbonates, which can remain as residues of the industrial process of alkalization of the cocoa nibs, which is usually employed to obtain a darker colour and milder taste of the chocolate (Herman et al., 2003).

Because of all those reasons, it is of primary importance, to utilize a suitable DNA extraction method. Therefore a peanut containing chocolate matrix was made to assess the applicability of the previously reported real-time PCR method (Scaravelli et al., *in press*) for the detection of peanut residues in chocolate. A milk chocolate matrix with a cocoa content of 28 % (Chocolate **G**, Table 1) was chosen because milk chocolate is the most popular amongst all types of chocolates (Wells, 1999) and can be eaten in a bar form or utilized to coat other food preparation such as biscuits, ice cream or sugar confectionery products.

Extraction additives, like skim milk powder or fish gelatine, are sometimes reported to improve protein extraction from complex food matrices (e. g., dark chocolate). The ELISA kit used in this study also includes this option in its protocol. Therefore the effect of such additives on the extraction of DNA from the chocolate matrix was investigated. Different aliquots of milk powder (0.5, 1.0, 1.5, 2.5 g) were added to the chocolate samples, before DNA extraction with the guanidine based extraction buffer. The other additive tested was gelatine that was suggested as extraction additive in the protocol of the peanut ELISA test kit. Gelatine was directly added to the guanidine based DNA extraction buffer. Chocolate samples containing peanut at a level of 10000 mg kg^{-1} were extracted in duplo and 5 µl of the undiluted DNA extracts was amplified or the DNA extracts were diluted 1:10 or 1:20 prior to PCR analysis. As reported by Gryson et al. (Gryson et al., 2007), quantification and or analysis by gel electrophoresis of DNA extracted from chocolate is not always consistent with the PCR results because of the possible presence of inhibitors or because of DNA fragmentation. Therefore no DNA quantification was applied in this study and a fixed aliquot

of pure or diluted DNA was added to the PCR reaction. Dilution of the DNA extract was applied in order to reduce the concentration of the PCR inhibitors allowing the PCR reaction to proceed (Germini et al., 2005) although this can reduce the method sensitivity. Alternatively, the influence of inhibitors of the PCR reaction like carbonates can be reduced by using a modified DNA extraction and a PCR buffer with an elevated Mg^{2+} content as proposed by Herman et al. (2003). But, since this might affect the specificity of the method due to non specific primer annealing (www.qiagen.com) this was not investigated here.

Figure 1 reports the effects of the use of additives during DNA extraction. When the results of the PCR analyses on undiluted DNA extracts are compared it is apparent that addition of gelatine prevents the detection of peanut, while the use of the two lower concentrations of milk powder do not decrease Ct values compared to the sample without extraction additives. Addition of larger amounts of milk powder had a clear detrimental effect as apparent from the increase in Ct values and the large standard deviations. In all cases the Ct values obtained for the spiked chocolate were higher than the Ct values around 27-28 that were reported for cookies with the same peanut content (Scaravelli et al., *in press*; *Chapter 3*). This is most likely caused by the presence of inhibitors that influence the enzymatic activity of the Taq polymerase. Dilution of the DNA extracts leads to a decrease of the standard deviation when samples without additives are analysed. But, it also shows a positive effect of both extraction additives. Moreover, using gelatine as additive resulted in Ct values comparable to those obtained after analysis of cookies with the same peanut content. This implies that the DNA extraction yield was not affected by the presence of chocolate matrix components. The use of gelatine during DNA extraction and dilution of the DNA extract was therefore applied to the analysis of all chocolate samples.

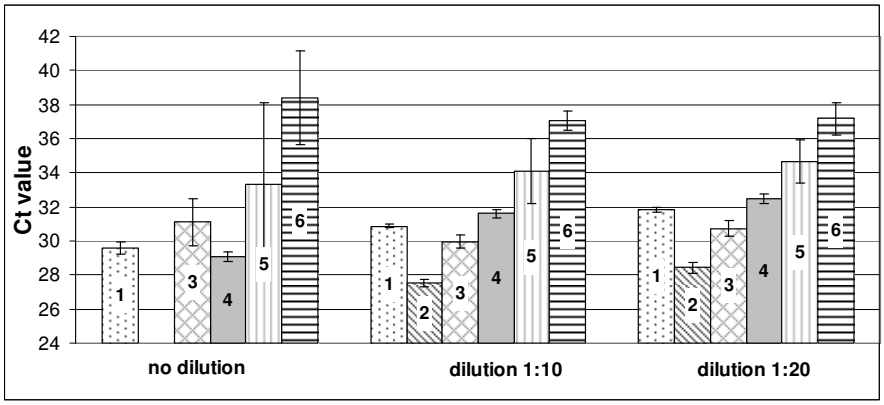

Figure 1: The effect of extraction additives on the amplification of peanut DNA. The data points are based on the mean of 4 cycle threshold values (two per each DNA extract) and the respective standard deviations are given. Chocolate containing peanut at a level of 10000 mg kg-1 was used for DNA extraction, while modifications of the extraction method were applied. (1: method without extraction additives; 2: addition of gelatine to the extraction buffer; 3, 4, 5, 6 : addition of respectively 0.5, 1, 1.5 or 2.5 g skim milk powder to each sample). Results were obtained by analysing pure DNA extracts or DNA extracts that were diluted 10 or 20 times.

Table 1: Specifications of the different types of chocolate used in this study.

Chocolate	cocoa solid (%)	cocoa butter (%)	milkfat (%)	total fat (%)
A	55.30			36.1
B	45.50	36.00	7.5	43.5
C	35.00	28.50	4.3	32.8
D	32.70	26.10	5.9	31.5
E	32.40	24.50	4	28.5
F	29.60	24.00	6.3	30.3
G	28.00	25.00	6	31

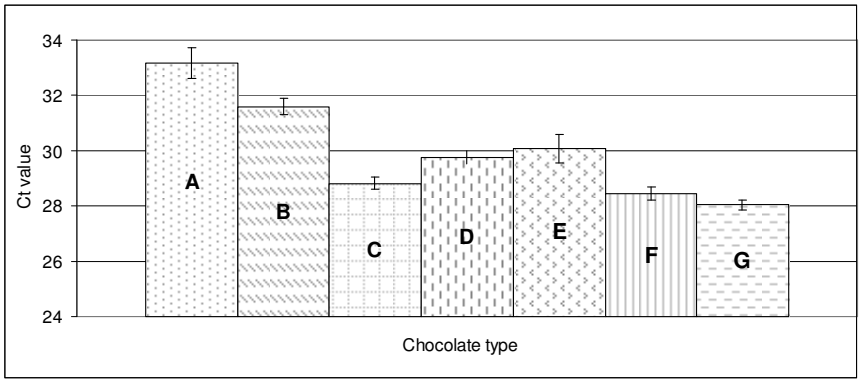

Figure 2: Amplification of DNA extracted from 7 different types of chocolate. The mean of 4 cycle threshold values (two per each DNA extract) and their respective standard deviation are given. Ct values were obtained after analysis of peanut spiked milk and dark chocolates with different amounts of total cocoa solids (Table 1).

As listed in the Codex Alimentarius (Codex Alimentarius, 2003), different types of chocolate can be distinguished by their "total cocoa solids content". For example, dark chocolate (≥ 35 %), sweet chocolate (≥ 30 %), milk chocolate (≥ 25 %) or family milk chocolate (≥ 20 %) can be distinguished. In order to evaluate the effect of the cocoa content on peanut DNA extraction and amplification, six milk chocolates with total cocoa solids contents ranging from 28 to 45.5 % and one dark chocolate with 55.3 % (Table 1) were spiked with peanut at a level of 10000 mg kg^{-1} and subsequently analysed by real-time PCR. Two replicates of DNA extraction were carried out and analysed after a 20 fold dilution of the DNA extract. This dilution was absolutely required to permit the amplification reaction in dark chocolate. The data presented in Figure 2 show the differences in Ct values obtained with the different chocolate materials. Although the peanut DNA amplification occurred in all samples analysed, the Ct values ranged from 33 for the chocolate with 55.3 % "total cocoa solids" to 28 for the chocolate with 28 % of "total cocoa solids". From the figure it is clear that in general a high content of "total cocoa solids" has a negative effect on peanut detection.

We conclude that the use of gelatine as extraction additive combined with a 20 fold dilution of the DNA extracts allowed amplification of peanut DNA from all chocolate matrices tested. However, a cocoa content of more than 35 % was shown to have a negative effect on amplification.

Characterization of a model chocolate matrix

The applicability of the real-time PCR method for the detection and quantification of peanut traces was further assessed by using the model milk chocolate matrix and a comparison with ELISA.
All different chocolates were analysed in duplicate with PCR C and the ELISA kit. In both cases gelatine was used as additive for protein or DNA extraction.
The peanut content in the chocolate material was estimated with the ELISA kit as described in the manual, by applying a calibration curve based on the protein standards provided in the kit. Peanut was detected in all different concentration levels, ranging from 10 mg kg^{-1} to 10000 mg kg^{-1}. For analysis with the real-time PCR method DNA extracts were diluted ten fold. Additionally DNA extracts that were diluted twenty fold were analysed. The PCR C assay gave a linear response within a concentration range of 10 mg kg^{-1} to 10000 mg kg^{-1} after applying either of the DNA dilutions. Therefore, this material was used to establish calibration curves.
However, prior to the establishment of calibration curves the homogeneity of the material was tested. Previously we have shown that both this real-time PCR method and the ELISA kit used in this study represent appropriate techniques for homogeneity testing (*Chapter 3*), therefore both were used to analyse the model chocolate matrix material. The chocolates containing peanut at a level of 10 or 100 mg kg^{-1} were analysed by both methods. Statistical analysis of variance (ANOVA) confirmed that with a 95 % probability, the milk chocolate materials could be considered homogeneous either after applying the DNA or the protein based detection method.
Having assessed that the material was homogeneous, it was used to generate two calibration curves with the real-time PCR C method, with the aim to interpolate samples with an unknown peanut content. For the interpretation of results obtained for milk chocolate samples, a calibration curved based on a 1:10 dilution of DNA extracts was established, while a curve based on a 1:20 dilution was established for the analysis of (dark) chocolate samples where a 1:10 dilution does not suffice. The characteristics of the calibration curves are listed in Table 2 and compared to the previously reported values for a calibration curve for cookies established with the same PCR method. Both curves related to chocolate show a PCR efficiency close to 100 %. Thus the efficiency does not seem to be affected by the chocolate matrix, despite the fact that chocolate is considered a problematic matrix due to its high content in phenolic compounds that could interfere either with DNA or protein extraction as well as their detection by PCR or ELISA (Gryson et al., 2004; Gryson et al., 2007; Porebski et al., 1997; Aljanabi et al., 1999; Khanuja et al., 1999, Katterman and Shattuck, 1993).

It is known from previous studies employing different ELISA kits (Poms et al., 2005; Whitaker et al., 2005) and PCR assays (Lipp et al., 2005; Gryson et al., 2004), that for both types of method repeatability was matrix-dependent. Therefore, an investigation into the effect that the chocolate matrix has on the repeatability of these two analytical methods was carried out. For a proper comparison of the data obtained with the two different methodologies, the relative standard deviations calculated on the signals collected by both real-time PCR and ELISA are shown in Table 3, and compared to the previously reported values for cookies as established with the same methods. For both food matrices analysed a decrease in peanut content caused an increase in measurement variability for both the real-time PCR and ELISA. Those results are consistent with the outcome of a validation study in which the same ELISA kit was tested (although there the CV % was determined on the interpolated values and not on the raw signals) (Poms et al., 2005). Compared to cookies, the analysis of chocolate samples is characterised by a higher source of variability for both methods, especially when the peanut content is low. Despite the fact that the CV values resulted in a similar trend for both matrices, the extent with which the matrices affected the repeatability of the two methods was higher for ELISA, with CV values averaging 5.3 % for cookies and 17 % for chocolate, compared to real-time PCR where those values were 1.2 % and 1.5 % respectively.

Table 2: Characteristics of the calibration curves of PCR C. ***Choco 10*** refers to DNA extracts from chocolate that were diluted 10 fold; ***Choco 20*** refers to DNA extracts diluted 20 fold; cookies refers to the characteristics of the method as applied to a cookie matrix that were reported previously (*Chapter 3*).

	cookies	**choco 10**	**choco 20**
Slope	-3.27	-3.61	-3.39
Intercept	41.07	42.08	42.03
PCR efficiency %	102.02	89.35	97.30
R-Squared	98.66	98.67	98.89

Table 3: Coefficient of variation (%) of the signals obtained by ELISA and real-time PCR after analysis of cookie and chocolate matrices with different peanut contents. Cookie data were reported previously (*Chapter 3*).

	ELISA		REALTIME PCR	
mg kg^{-1}	cookie	chocolate	cookie	chocolate
10	5.1	40.4	2.1	2.5
100	6.9	18.5	1.0	1.7
1000	6.4	5.5	0.8	1.0
10000	3.0	3.7	1.0	0.9
CV % tot	5.3	17.0	1.2	1.5

Market survey

In a recent study a total of 315 different biscuits and 254 different chocolates were purchased in 10 European countries and analysed with a lateral flow device (dipstick) and a sandwich-type ELISA (Pele at al., 2007). The aim of that study was to evaluate the analytical data obtained with the two methods and compare it with the information provided on the labels of the food products. In the present work we aimed to confirm the reported analytical data by analysing samples of this survey with the real-time PCR assay developed to analyse such samples. All cookie and chocolate samples that had tested positive with ELISA and or dipsticks were analysed with the real-time PCR method. In addition to this a number of samples that had tested negative were included in our study. A total of 105 chocolate and 103 cookies samples were analysed by real-time PCR.

In the current legislation (FALCPA, 2004; European Parliament and Council, 2000, 2003, 2007) no threshold limits for peanut are mentioned below which a food product is considered either safe for consumption by allergic consumers, or does not have to declare the allergenic ingredient on the label. Therefore a qualitative analysis yielding a "yes" or "no" response with regard to the presence of peanut traces in a food product is required.

Analysis of the food samples with a dipstick results directly in a qualitative response, since the appearance of the "positive line" indicates the presence of the target protein, while absence of this line means that the target can not be detected. ELISA analysis is characterised by a numerical output, but any signal higher than the LOD identifies a positive sample. In case of the BioSystems peanut allergen test kit, this threshold was set at 0.7 mg kg^{-1} (Pele et al., 2007).

In case of the real-time PCR method a qualitative analysis requires a Ct value to be set as LOD.

Table 4: Criteria used to assign positive and negative analytical results to the samples analysed by real-time PCR C. The criteria are based on the number of analyses returning Cycle threshold values, after triplicate analysis (Ct score). Q: quantifiable sample, for which the respective calibration curve can be applied;

LOD criteria		
Ct score	Ct ≤ 40	40<Ct≤45
3 / 3	Q	POS
2 / 3	Q	POS
1 / 3	POS	NEG

Therefore, both calibration curves for quantification and LOD values were defined in this study. The three calibration curves listed in Table 2 were applied for the interpolation of Ct values of unknown samples and to estimate the peanut content a range between 10 and 10000 mg kg^{-1}. Although quantification could only be applied within that range, we have previously shown that the method can be used in a qualitative fashion when Ct values above 40, but below 45 are considered

(*Chapter 3*). For the interpretation of results from the market survey samples that were analysed by real-time PCR such Ct values were indeed considered to identify positive samples. Nevertheless, when after triplicate analysis only a single Ct value above 40 was obtained the sample was not considered positive. The criteria applied for the interpretation of the analytical results are listed in Table 4.

The qualitative results obtained by real-time PCR were compared with the ones obtained with ELISA and dipstick analysis. The results of this are presente d in Figure 3 and Figure 4.

As reported earlier, there is a relatively good agreement with ELISA and dipstick analysis of

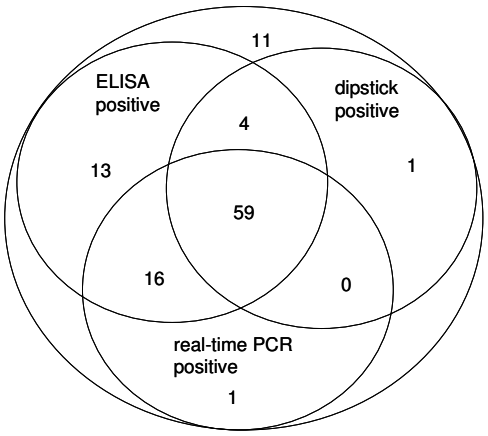

Figure 3: Venn diagram showing the number of **chocolates** yielding negative or positive test results by either ELISA, dipstick or real-time PCR analysis. Total number of samples: 105.

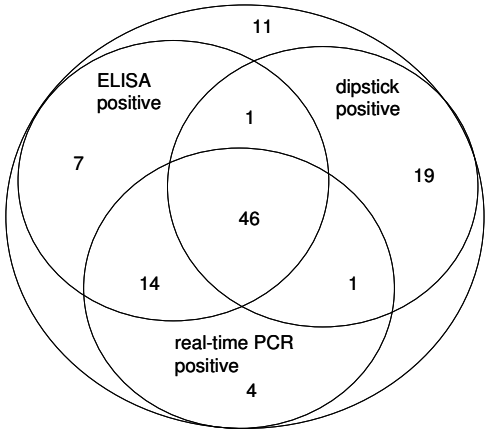

Figure 4: Venn diagram showing the number of **cookies** yielding negative or positive test results by either ELISA, dipstick or real-time PCR analysis. Total number of samples: 103.

chocolate samples, although ELISA seemed to be more sensitive since it identified more positive samples (Pele et al., 2007). Interestingly, the same holds true for ELISA and real-time PCR, the latter method identified only a single positive sample that did not yield a positive result after ELISA analysis, while ELISA identified 17 positive samples that did not yield a positive result after PCR analysis (Figure 3). In a total of 75 samples the presence of peanut traces was confirmed by both ELISA and real-time PCR analysis. Four samples were shown to contain peanut after analysis by the immunological methods, but not after real-time PCR analysis. There are two possible explanations for this. Either the food products contain mainly peanut protein and therefore no peanut DNA can be detected, or alternatively the immunological methods yielded false positive results caused by the cross reactivity of the antibodies used with one or more matrix components. The latter explanation is plausible since both the ELISA and the dipsticks used employ the same antibodies. Figure 4 shows the results obtained after analysis of the cookie samples. As reported previously, the agreement between results obtained after ELISA and dipstick analysis is not as high as that observed for the chocolate samples (Pele et al., 2007). Forty-one samples were identified as

positive with only one of those methods; while in slightly more samples (47) peanut traces were detected by both methods. Interestingly, when ELISA and PCR analysis are considered a better agreement between results was observed (Figure 4 and Table 5). Twelve samples were identified as positive with only one of those methods, while for 60 samples peanut traces were detected by both methods.

Taken together, around 85 % of all samples analysed resulted in the same qualitative response after ELISA and real-time PCR analysis (Table 5). It is noteworthy that the agreement between the immunological methods is not as high, despite the fact that the dipsticks and ELISA kit used detect the same target molecule (Ara h 1) and employ the same antibodies. Differences in the sensitivity or methodology between both immunological methods have been implied to cause the difference in their responses (Pele et al., 2007). Surprisingly ELISA and real-time PCR resulted in a better agreement although the target molecules were of a completely different nature.

Table 5: Qualitative agreement of analytical results obtained by ELISA, dipstick analysis and real-time PCR. The qualitative agreement is expressed in absolute numbers or as a percentage of the total number of cookies or chocolates analysed.

	COOKIES		**CHOCOLATES**	
	units	**%**	**units**	**%**
Total samples analysed	*103*		*105*	
ELISA-RTPCR	*90*	**87**	*87*	**83**
DIPSTICK -RTPCR	*63*	**61**	*83*	**79**
ELISA-RTPCR-DIPSTICK	*57*	**55**	*70*	**67**

Table 6: Quantitative agreement of analytical results obtained by ELISA and real-time PCR analysis. The qualitative agreement is expressed in absolute numbers and indicates that analytical results obtained with both methods showed less than 30 % variation. Numbers in brackets indicate the number of samples for which only qualitative results were obtained.

	mg kg^{-1}											
	negative			**0.7 - 10**			**10 - 20**			**>20**		
	ELISA	**RTPCR**	***Agreement***	**ELISA**	**RTPCR**	***Agreement***	**ELISA**	**RTPCR**	***Agreement***	**ELISA**	**RTPCR**	***Agreement***
COOKIES	35	38	***30***	27	15 *(+12)*	***9***	2	5	***1***	39	33	***32***
CHOCOLATES	13	29	***12***	30	15 *(+12)*	***4***	12	13	***4***	50	36	***36***

In addition to providing a qualitative assessment, both the ELISA and real-time PCR methods were employed to obtain an estimation of the peanut content in the food samples based on the calibration curves. Amongst the cookie samples tested 65 were positive by real-time PCR analysis but, only 31 of them were quantifiable, with an estimated peanut content in the range of 10 to 10000 mg kg^{-1}. In 27 samples the estimated peanut content was lower than 10 mg kg^{-1} (extrapolation of the calibration model) or simply positive according to the criteria listed in Table 4, while in seven samples the estimated peanut content was clearly higher than 10000 mg kg^{-1}. The last group contained six food

products that declared peanut as an ingredient and one with no reference to peanut or nut on the label. For chocolate, 76 samples tested positive by real-time PCR analysis while 44 of them were quantifiable according to the criteria mentioned above. Four samples that declared peanut as an ingredient had an estimated peanut content clearly exceeding 10000 mg kg^{-1}. To assess the quantitative agreement between real-time PCR results and ELISA results the samples had to be grouped according to their estimated peanut content. The estimation of the peanut content as determined by ELISA falls within the quite narrow working range of 0.7 to 20 mg kg^{-1}. In contrast to this, real-time PCR allowed for quantification up to 10000 mg kg^{-1}. Therefore, all samples with a peanut content estimation exceeding 20 mg kg^{-1} as determined by real-time PCR can considered to be in agreement with ELISA if with the latter method more than 20 mg kg^{-1} was detected (although with ELISA no numerical results were obtained). Except for a single cookie sample this was indeed found to be the case. A direct comparison of quantitative results is allowed within the very small range of 10 to 20 mg kg^{-1}. Since only a few samples are within this range we also considered the range of 0.7 to 10 mg kg^{-1} where the peanut content could be estimated by extrapolation of the real-time PCR calibration curve. To take factors like uncertainty of measurements and the very different nature of both methodologies into account we allowed a variation of 30 % between the estimated peanut levels as determined by ELISA and real-time PCR. When the variation was below 30 % for a given sample we considered the quantitative results to be in agreement. Table 6 shows that for half of all cookie samples for which real-time PCR results were within the range of 0.7 to 20 mg kg^{-1} the estimated peanut content was in agreement with the ELISA results. For chocolate this was the case for around 30 % of the samples.

After analysis of chocolate 17 samples tested positive by ELISA while returning negative responses after real-time PCR analysis. A possible explanation for this might lie in their high cocoa content, since many of those samples contained “total cocoa solids” at levels exceeding 40 %. As we have shown above such high levels of cocoa solids have a detrimental effect on the detection of peanut residues by real-time PCR.

Analytical results and food labels

The study reported by Pele et al. focussed on the comparison of ELISA analysis and the information on the labels of the food products. For this a distinction was made between products declaring peanut as an ingredient, products with a precautionary warning and products that did not mention peanut or nut on the label. Precautionary warnings were subdivided in ‘‘may contain’’ type of labels

and ‘‘present in environment’’ type labels (e.g. ‘‘this product is made on a line that also handles peanut’’ or ‘‘this product is made in a factory that also produces peanut-containing products’’) (Pele et al., 2007). Such precautionary warnings either referred to peanut or to the more generic term nuts (van Hengel, 2007). In the current study the results obtained by real-time PCR analysis were compared with the information on the labels of the food products analysed and with the ELISA and dipstick results. It has to be stressed that the PCR analysis was aimed at a confirmation of the positive results obtained by ELISA and dipstick analysis, and therefore the data on the comparison as presented in Table 7 are not representative of the complete set of samples that was purchased. Of the cookies that declared peanut as an ingredient only 7 tested positive after ELISA analysis, questioning the presence of peanut in those samples (Pele et al., 2007). After real-time PCR analysis two more samples tested positive (Table 7), indicating that those two samples can indeed contain peanut as an ingredient. For cookies of all types of labelling that were differentiated, the number of samples that tested positive with the real-time PCR method was very similar to the ones that had tested positive with ELISA. For chocolate almost the same number of positive samples were detected with both methods after analysis of food products carrying a label without any reference to nut or peanut, but ELISA identified more positive samples carrying a precautionary warning (Table 7).

Table 7: Relationship between the type of labelling and positive analytical results. Cookies and chocolates from the market survey, were analysed with ELISA, dipstick and real-time PCR.

COOKIES		SAMPLES	**ELISA**	**DIPSTICK**	**RT PCR**
		units	positive	positive	positive
	peanut	15	7	7	8
INGREDIENT	nut	1	0	1	1
	peanut & nut	1	0	0	0
	peanut	13	7	10	7
"MAY CONTAIN…"	nut	7	3	6	2
	peanut & nut	8	8	7	8
	peanut	7	6	5	4
"PRESENT IN ENVIRONMENT"	nut	0	0	0	0
	peanut & nut	3	3	2	3
"NO REFERENCE"		48	35	29	33

total COOKIE samples: 103

CHOCOLATES		SAMPLES	**ELISA**	**DIPSTICK**	**RT PCR**
		units	positive	positive	positive
	peanut	5	4	4	4
INGREDIENT	nut	0	0	0	0
	peanut & nut	0	0	0	0
	peanut	38	33	24	29
"MAY CONTAIN…"	nut	10	8	2	5
	peanut & nut	35	31	23	26
	peanut	0	0	0	0
"PRESENT IN ENVIRONMENT"	nut	1	0	0	0
	peanut & nut	1	0	0	0
"NO REFERENCE"		15	13	11	12

total CHOCOLATE samples: 105

For the allergic consumer the amount of peanut ingested determines whether an allergic reaction occurs and how severe this reaction is. Therefore it is important to determine the peanut content in the analysed samples. By utilizing real-time PCR the peanut content was estimated and compared to the information on the label of the food products. In cookie samples of products carrying a precautionary warning the highest peanut content detected was 7700 mg kg^{-1}, while for chocolates this was 1800 mg kg^{-1}. Unfortunately, even higher levels were detected in food products with no reference to peanuts or nuts on their labels with the detection of peanut at a level just exceeding 10000 mg kg-1 both for cookies and chocolate.

Conclusions

The detection of hidden allergens in food products represents an important challenge for the scientific community. Many commercial test kits for allergen detection that are either based on DNA or protein detection are currently on the market. Their reliability is normally assessed by investigating their performance on the analysis of a very limited number of (model) matrices developed for this need by each research group. This can not represent the complexity and variety of matrices that are encountered by the analysis of market samples. The availability of multiple methods for the detection of peanut residues allows conformation of analytical results. When DNA based detection methods are used to confirm results obtained with protein based methods both should yield comparable results. Recently three real-time PCR methods were compared with two commercial ELISA kits by analysing 25 different peanut containing samples (Engler-Blum et al., 2007), while also previous studies had focused their attention on the analysis of market samples (Stephan and Vieths, 2004; Watanabe et al., 2006; Piknova et al., 2007). However, in those studies a peanut content as determined by ELISA is usually compared with a raw signal (Ct value) obtained by real-time PCR.

In this study we aimed to confirm analytical results obtained by ELISA in a large market survey, the real-time PCR method developed for the detection of peanut traces in cookies was extended to include chocolate matrices. For this extension a modification of the DNA extraction method was required. The use of gelatine and dilution of the extracted DNA prior to PCR analysis was show to be required to cope with PCR inhibition, which was shown to increase with increasing cocoa content. This supports the notion that there is no “universal” DNA extraction method that can be applied to any type of food matrix (Di Bernardo et al., 2007). Although chocolate was confirmed to be a problematic matrix, two calibration curves were established to estimate the peanut content of

unknown samples with the use of a model chocolate matrix that was spiked with the peanut test material IRMM-481. Having developed calibration models applicable for two different types of products from the confectionary industry (biscuits and chocolate), samples that had previously been analysed with an ELISA assay and a lateral flow device were tested with the developed real-time PCR method.

With regard to the current legislation (FALCPA, 2004; European Parliament and Council, 2000, 2003, 2007) a qualitative assessment of the presence or absence of peanut residues is of prime interest. Therefore, the fact that 85 % of the ELISA results could be confirmed by real-time PCR stresses the usefulness of both techniques that clearly complement each other. Although a quantitative comparison of real-time PCR and ELISA was hampered by the fact a large number of samples with a peanut content exceeding 20 mg kg^{-1} were not quantified with the latter method, 18 samples showed a qualitative agreement of the results obtained with both methods. This is remarkable since a criterion of only 30 % variation was allowed for a quantitative agreement, while both methods target markers of a completely different nature.

The availability of multiple methods for the detection of peanut traces in food products is of paramount importance to protect the peanut allergic individual, and has identified a multitude of chocolates and cookies for which peanut was not an ingredient that tested positive. The detection of sometimes very high levels of peanut in market samples stresses the need for such methods and for the analysis of food products that might endanger the health of allergic individuals.

Details on the market survey results can be found in the Annex I.

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Chapter 5

A PNA-Array platform for the detection of hidden allergens in foodstuffs

A PNA array device has been designed and implemented to be used in combination with a duplex PCR in order to simultaneously investigate the presence of traces of potentially allergenic hazelnut and peanut in food products. A PNA probe for each target amplified by the duplex PCR was designed, synthesised and characterised. The PNA probes were then deposited on commercial slides in order to build a PNA array platform to be used for recognising PCR products.

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Introduction

During the recent years food allergy prevalence has emerged as a serious public health issue affecting around 1 % of world adult population and up to 2.5 % of children population respectively (Jackson et al., 2003). Food allergens are proteins or glycoproteins with molecular weight ranging from 10 to 70 kDa and although any protein is potentially allergenic, the most severe adverse reactions to food are associated with the consumption of a small number of products classified in eight main food groups responsible for about 90 % of all IgE mediated food allergies: cow's milk, eggs, fish, crustaceans, peanuts, soybeans, tree nuts and wheat. Since anaphylactic reactions are reported to take place even after consumption of very low amounts of allergenic proteins (Yunginger et al., 1988), the presence of hidden allergens in food products, not listed on the label, is a great problem causing concern in sensitive people. To face the problem, avoidance of the allergenic food or component is at present the only practical way for allergic patients; nevertheless hidden allergens cause serious concern even after this precaution. In this context it is of great importance the new directive of the European parliament (European Parliament and Council, 2003) regarding the comprehensive labelling of allergenic food ingredients, in which the declaration of all the major allergenic ingredients (Annex IIIbis: cow's milk, eggs, fish, crustaceans, peanuts, soybeans, tree nuts, wheat, celery, mustard, sesame seeds and sulphites), regardless of the total amount in the final product, is made compulsory. Open is still the problem of threshold levels of the allergens, i.e. the minimum quantity of a protein which elicits an allergic reaction.

Among the categories of allergenic foods, peanuts and tree nuts are probably the ones most likely to be responsible for contamination of other materials during food manufacturing on account of their widespread use as ingredients in many food products, and for this reason they should be considered as potential source of hidden food allergens. Moreover, peanuts and tree nuts are not only responsible for 1.1 % of all the allergic reactions in the USA (Sicherer et al., 1999), but are becoming an emerging problem also in Europe (Ewan, 1996) probably on account of the increased consumption.

Although the determinations of allergens in food (Besler, 2001; Poms et al., 2004) is mainly based on immunological methods via the use of either specific IgE from human serum or mono and polyclonal antibodies of animal origin, a sensitive indirect determination of the possible presence of food allergenic proteins can be performed targeting their specific cDNA via PCR methods. This approach has already been used for the indirect detection of specific food allergens in various matrices such as wheat (Köppel et al., 1998), olive (Aché et al., 2002), apple (Son et al., 1999) and hazelnut (Holzhauser et al., 2000; Herman et al., 2003) and seems to be suitable in particular when

applied to matrices in which protein concentration is low (e.g. hidden allergens) or proteins are partially degraded by processing. However, PCR systems can sometimes lead to ambiguous interpretations on account of the low specificity of the priming sequences or of "carry-over" contaminations producing false positives (Quist and Chapela, 2001). Although the latter can be avoided by applying good laboratory practices, in all cases of ambiguous interpretations it is advisable to confirm the identity of the PCR amplicons. This may be done in different ways including sequencing of the amplicons, using nested PCR or using probes specifically hybridizing target DNA sequences. Among these, particularly useful turned out to be peptide nucleic acids (PNAs), oligonucleotide analogues in which the sugar-phosphate backbone has been replaced by a pseudopeptide chain of *N*-aminoethylglycine monomers, endowed of a higher sequence specificity (Egholm et al., 1993; Jensen et al., 1997; Dueholm and Nielsen, 1997).

In two recent works we successfully applied PNA probes to the post PCR detection of a specific DNA sequence identifying the presence of hazelnut in foodstuffs via HPLC (Germini et al., 2005), and of GM soybean via an array device (Germini et al., 2004).

In the present work we describe the implementation of a PNA array for the detection of hazelnut and peanut in various foodstuffs after the simultaneous amplification of their DNA by a duplex PCR designed and developed for the purpose. The method here proposed allows to detect the presence of DNA coming from two potential sources of hidden allergens (i.e. hazelnut and peanut) and to confirm simultaneously their identity via hybridization with PNA probes.

Materials and methods

Commercial samples

A set of ten commercial foodstuffs, including breakfast cereals, snacks, biscuits and chocolate, were purchased in randomly chosen food stores.

DNA extraction.

DNA extraction was performed using the commercial kit *Wizard® Plus Minipreps DNA purification system* (Promega), opportunely modified as described in a previous work (Germini et al., 2005). The modifications can be summarized as follows: once the DNA was extracted and purified following the instructions provided in the commercial kit, it underwent a further purification step by

addition of 0.1 volumes of 3 M NaOAc (pH 5.2) and two volumes of cold 95 % ethanol, followed by incubation at –70°C for 30 minutes. After precipitation the DNA was then pelleted by centrifugation at 12000 rpm for 15 minutes at 4 °C. The DNA pellet was washed with 80 % ethanol and incubated at room temperature for 10 minutes, then centrifuged for 5 minutes at 12000 rpm; the DNA pellet was finally dried and then dissolved in 100 µl of water. The DNA concentration was evaluated by UV absorption at 260 nm; 30 ng/µl stock solutions were prepared by dilution with bidistilled water.

Duplex PCR.

A duplex PCR system simultaneously targeting a DNA sequence characterizing the Cor a 1.0301 isoform of the hazelnut allergen (GenBank acc. number Z72440.1) and one characterizing the Ara h 2 peanut allergen (GenBank acc. number L77197.1) was designed, developed and validated. The primer sequences chosen for the analyses are reported in table 1.

The PCR primers to be incorporated in the single DNA strand to be hybridised with the PNA array (Cor a1_74 and Ara h2_455) were labelled with a Cy5 fluorophore at their 5' end.

The samples for PCR screening were amplified using the method described below, whereas all samples to be used on the array platform underwent a double amplification: the first step was used to amplify the target sequences, while the second step was an unbalanced PCR used to selectively amplify the target DNA single strands to be hybridised on the array.

Polymerase chain reactions were performed in a final volume of 50 µl. All the reagents were supplied by *Euroclone* and the primers purchased from *Thermoelectron*. The concentration of the reagents in the first PCR were: 1x reaction buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 0.1 u/µl of hot start Blue Taq, 150 ng of DNA. The concentrations for the second step of the unbalanced PCR were the same, apart from the primer concentrations brought to 0.2 µM for the oligonucleotide priming the non target DNA strand, and 2.0 µM for the oligonucleotide priming the target DNA strand (Table 1). The PCR was carried out in a PCR-sprint thermal cycler (*Thermohybaid*) using the following conditions: 1 cycle of DNA denaturation and Blue Taq activation at 95 °C for 5 minutes; 40 cycles consisting of DNA denaturation at 95 °C for 50 seconds, primer annealing at 60 °C for 50 seconds and elongation at 72 °C for 50 seconds; one step of final elongation at 72 °C for 5 minutes.

Table 1

Table 1. Primers and PNAs used for the assay. (L: (2-aminoethoxy) etheroyl acid spacer group)

Primer	Primer sequence (5'-3')	Copied strand	Target	PNA sequence ($H-NH_2$)
Cor a 1_74	TAG ATT CCG ACA ACC TCA TCC	Target	Hazelnut	LL – ATG ATT TCA ATG CTC
Cor a 1_229	CAC AAA ACG TAC AAC TCC TTG G	Non target		
Ara h 2_455	GGC GGC AGA GAC AGA TAC TAA	Target	Peanut	LL – CGA TCA ACA CAT TCA
Ara h 2_655	GAC AAC GCC ATA AAA GCA CTC	Non target		

PNA array preparation

PNA design

The PNA sequences were first checked to minimize any secondary structure which would result in a loss of hybridization efficiency, using the online available program Mfold (version 3.1) (Zuker, 2003). The sequence specificity of the probes was first evaluated using the BLAST homology search system from DDBJ (http://www.ddbj.nig.ac.jp/E-mail/homology.html) and then both probes were checked in order to avoid hybridization with any other non-target region among those amplified by the duplex PCR, by aligning the PNA sequences to the DNA sequences of the other amplified products. The sequences chosen for the analyses are reported in table 1.

PNA synthesis

The PNA oligomers were synthesized by solid-phase synthesis on a 433A Peptide Synthesizer (Applied Biosystems) using the BOC strategy and HBTU/DIEA coupling, as described in a previous work (Lesignoli et al., 2001). Swelling, downloading and cleavage of the PNAs from the resin were done manually. Two 2-(2-aminoethoxy)ethoxyacetic acid spacers were added at the N-terminus of the PNA to link it to the solid surface of the array. The crude products were purified by reversed phase HPLC using a Phenomenex C18 peptide column (3 μm, 250 mm × 10 mm) with a binary gradient (flow rate: 4mL/minutes); eluent A: water /TFA = 100 : 0.2; eluent B: water/acetonitrile/TFA = 60 : 40 : 0.2; detector UV (260 nm). The purified products were characterized by Electrospray Ionization-mass spectrometry (ESI-MS).

Array preparation

"CodeLink™ Activated Slides" (Amersham Biosciences) were used as solid supports to which the amino-terminal group of the PNA probes were covalently linked. The deposition of the probes was carried out using a GMS 417 Arrayer (Genetic Microsystem) with a pin-and-ring deposition system.

The manufacturer instructions for the deposition protocol were slightly changed in order to comply with the special requirement of the chemical structures of PNAs: in particular a 100 mM carbonate buffer (pH 9.0) containing 10 % acetonitrile and 0.001 % sodium dodecyl sulphate (SDS) was used as deposition buffer. Moreover, after every deposition, the pin-and-ring system was purged with water for 10 seconds and further washed with acetonitrile/water (1:1), in order to avoid dragging of the probes in subsequent depositions. The probes were coupled to the surface and the remaining reactive sites were blocked by leaving the slides in a humid chamber (relative humidity 75 %) at room temperature for 12 hours, followed by immersion in a glass rack containing a 50 mM solution of ethanolamine, 0.1M TRIS, pH 9, prewarmed at 50 °C, for 30 minutes. The slides were washed twice with bidistilled water at room temperature and then slowly shaken for 30 minutes in plastic tubes containing a 4X salinesodium citrate (SSC) solution and a 0.1 % SDS buffer prewarmed at 50 °C. Each slide was then washed with bidistilled water at room temperature and centrifuged in a plastic tube at 800 rpm for 3 minutes. Slides were then ready to undergo the hybridization protocol or could be stored in a desiccated chamber for future use. It has to be remarked that, since a fluorescent control probe (CP sequence: NH_2–$(AC)_{11}$–Cy5) was deposited to check the efficiency of the deposition step, all the previously described operations were carried out away from direct light in order to prevent degradation of the Cy5 fluorophore.

Array analysis

Sample hybridization

DNA samples to be tested were prepared by diluting 50 µl of the PCR product to a final volume of 65 µl and a final concentration of 4X SSC and 0.1 % SDS buffer. Hybridization was performed by loading the samples to "in situ frame" chambers (Eppendorf, 0030 127.510) and leaving the slides under slow shaking for 2 hours at 40 °C. After the hybridization step all the slides were treated individually to prevent cross contamination. The slides were washed under slow shaking for 5 minutes at 40 °C with a 2 X SSC, 0.1 % SDS buffer prewarmed at 40 °C, followed by treatment for 1 minute with 0.2 X SSC and for 1minute with 0.1X SSC at room temperature. The slides were then spin-dried at 1000 rpm for 5 minutes. It has to be noticed that all post hybridization steps were performed in a dark environment to prevent degradation of the Cy5 fluorophore used to label the target sequences.

Image acquisition

The fluorescent signal deriving from hybridization was acquired using a GMS 418 Array Scanner (Genetic Microsystem) at λex = 646 nm and λem = 664 nm. In order to correctly compare the hybridization data, all the images reported were acquired with laser power = 100 and photomultiplier gain = 40.

Results and discussion

The primer pairs chosen for the development of the duplex PCR, assessed for their specificity during the design step by alignment in the online available databases, were then individually tested with real standard samples in order to verify their ability to amplify only their specific targets. For this reason the primer pairs designed for the detection of hazelnut and peanut were individually tested with a panel of 15 in-house standard food matrices composed by nuts (brazilian nut, almond, hazelnut, pistachio and walnut), legumes (lupine, chickpea, peanut, pea and soybean) and cereals (barley, maize, wheat, oat and rice), using the PCR method developed, as described in the material and methods section. In order to avoid false negative results and to confirm the presence of vegetable amplifiable DNA, all the standards, once extracted, were tested using a protocol for the amplification of different non-coding regions of chloroplast DNA (Taberlet et al., 1991). The results (data not shown) indicated that a positive amplification could be observed only when the designed primer pairs were applied to their specific targets and no amplification was observed for non target matrices. The length of the amplicons obtained was found to be consistent with the design of the single PCRs: 156 bp for hazelnut and 201 bp for peanut.

The limits of detection for the single and duplex PCR designed were then evaluated using subsequent dilutions of stock solutions of their target DNA ranging from 150 ng to 1 pg. Detection limits for the single PCRs were found to be 5 pg (data not shown), while the duplex PCR system, in agreement whit what already known in the literature (Matsuoka et al., 2000), was found to have a lower sensitivity, being able to amplify the target sequences down to 50 pg of target DNA (Figure. 1). The limit of detection for the PNA probes was assessed by hybridization of the PNA designed to target hazelnut with a synthetic 15mer oligonucleotide complementary to the probe and labelled with a Cy5 fluorescent group. The same set of probe concentrations (50, 40, 30, 20 and 10 □M) was spotted on ten slides which were then individually hybridized with concentration of the target oligonucleotide corresponding to 0.8 μM, 0.4 μM, 0.2 μM, 0.1 μM, 50 nM, 25 nM, 12.5 nM, 6.75

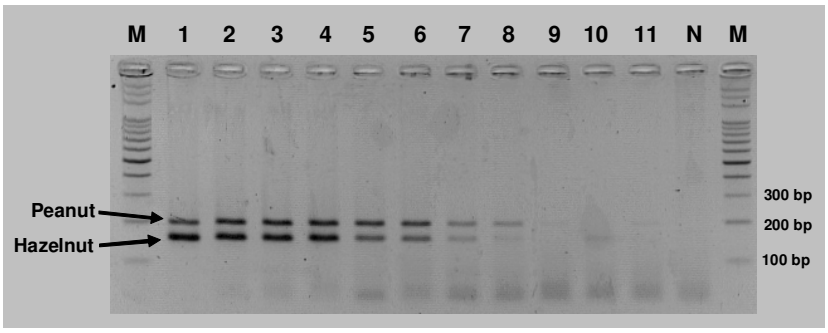

Figure 1: Limits of detection of the duplex PCR for the detection of hazelnut and peanut. Hazelnut amplicon size: 156 bp; Peanut amplicon size: 201 bp. Samples were prepared with concentration of the target DNAs corresponding to: 150 ng (1), 50 ng (2), 10 ng (3), 5 ng (4), 1 ng (5), 500 pg (6), 100 pg (7), 50 pg (8), 10 pg (9), 5 pg (10), 1 pg (11). M: 100 bp size marker. N: negative control.

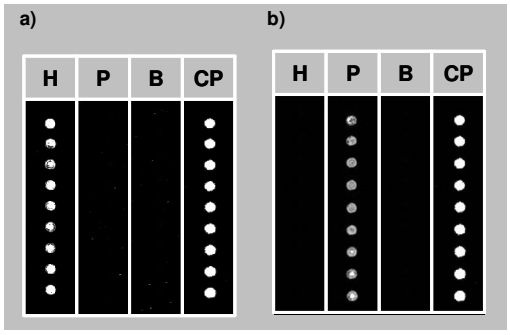

Figure 2: Evaluation of the PNA specificity. The arrays were tested with the PCR product deriving from amplification of DNA extracted from hazelnut (a) and peanut (b). H: hazelnut PNA; P: peanut PNA; B: blank lane; CP: Cy5 labelled control probe.

nM, 1 nM, 0.01 nM. The best hybridization signal (data not shown) in the whole range of target concentrations was observed for the 30 μM PNA, while no significant improvement was observed by increasing the PNA concentration. A statistically significative enhancement of the fluorescent signal (more than ten times the signal/noise ratio) was detected down to 1nM which can then be assumed as limit of detection of the array system. Although the oligonucleotide cannot completely mime the behaviour of a longer single stranded DNA, such as those resulting from amplification, these results were taken as indicative for choosing the concentration of the PNA probes to be spotted.

The array device was prepared by depositing nine replicates for each probe to be coupled, in three parallel lines corresponding to: H) 30 μM solution of the PNA for the detection of hazelnut; P) 30 μM solution of the PNA for the detection of peanut; CP) 30 μM Cy5 labelled control probe to control the efficiency of the deposition step.

In order to test the selectivity of hybridization, the PNA array platform was tested with two samples amplified from hazelnut and peanut standards respectively. The samples were prepared by double amplification as described in the "Materials and Methods" section, in order to produce a consistent amount of the target single strands. The samples were individually hybridized on two different arrays according to the protocol and then their images were acquired via a fluorescent reader. The results show that the PNA probes were able to selectively recognize the presence of hazelnut (Figure. 2a) and peanut (Figure. 2b) respectively.

In order to evaluate the applicability of the PNA array method to the detection of traces of hazelnut and peanut in foodstuffs, a panel of commercial food products, purchased on randomly chosen food stores, was chosen in order to test their content of hazelnut and/or peanut as claimed on the label, or for their possible presence as result of accidental contamination during manufacturing (table 2). Out of ten selected products, the presence of hazelnut as ingredient was declared in five samples and of peanut in one sample, while the possible presence of traces of tree nuts and peanuts as a result of manufacturing carryover was declared in three samples.

All samples were preliminarily tested with the duplex PCR method in order to evaluate their composition and to compare the results with what observed with the subsequent PNA array test. Almost all the selected samples, a part from sample 6, contained hazelnut at various levels, while only sample 3 contained both hazelnut and peanut (Figure. 3). This means that traces of hazelnut were found in four samples which didn't report hazelnut among the ingredients (samples 1, 3, 5, 9), two of which did not declared it as possible contaminant (samples 3 and 5); no contamination was found for peanut.

Sample 1 and sample 3 were then selected to be tested on the PNA array as representative for the analyses of hidden allergens. In fact: sample 1, in which the presence of hazelnut was declared as possible contaminant, was found to contain hazelnut; sample 3, in which the presence of peanut as ingredient was declared but not the presence of hazelnut either as ingredient or as possible contaminant, was found to contain both peanut and hazelnut.

Table 2: Commercial foodstuffs tested by PNA-arrays

Sample	Product	Declared content
1	Breakfast cereals	Possible traces of tree nuts and peanut
2	Breakfast cereals with chocolate and hazelnut	Hazelnut
3	Muesli snack with milk chocolate	Peanut
4	Muesli snack with cocoa	Hazelnut
5	Snack with cereals and cocoa	not present
6	Cocoa and vanilla wafer	not present
7	Hazelnut wafer	Hazelnut
8	Biscuit topped with cocoa cream and milk chocolate	Hazelnut. Possible traces of other tree nuts and peanut
9	Milk chocolate	Possible traces of tree nuts and peanut
10	Chocolate cream with hazelnut	Hazelnut

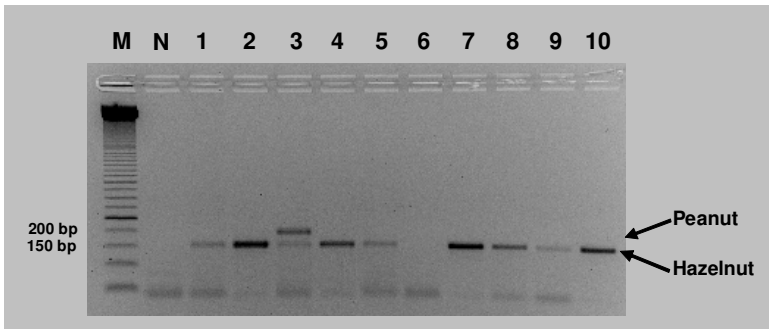

Figure 3: Duplex PCR for the detection of hazelnut and peanut applied to commercial foodstuffs, as reported in table 2. Hazelnut amplicon size: 156 bp; Peanut amplicon size: 201 bp. M: 50 bp size marker. N: negative control

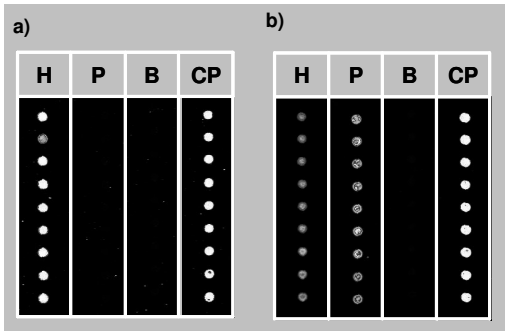

Figure 4: PNA array analyses for the detection of hazelnut and peanut applied to commercial foodstuffs (the numbers refer to the products reported in table 2): sample 1(a) and sample 3 (b). H: hazelnut PNA; P: peanut PNA; B: blank lane; CP: Cy5 labelled control probe.

The samples selected were doubly amplified, and then hybridized as described in the "Materials and Methods" section. The results obtained with the PNA array device were in agreement with those previously observed with the duplex PCR: sample 1 gave raise to a strong hybridization signal only on the first lane, indicating the presence of hazelnut in the tested product (Figure. 4a), whereas in sample 3 a strong fluorescent signal was observed on the second lane and a weaker signal was also observed for lane one, indicating the simultaneous presence of peanut and hazelnut in the tested product (Figure. 4b).

Conclusions

A new duplex PCR method for the detection of hidden allergens in foodstuffs was developed and tested. Its sensitivity was found to be comparable with similar methods published for other applications and was evaluated to be 50 pg of target DNA when using standards. Its applicability to commercial samples for screening purposes was also assessed by testing a number of selected products. It has to be noticed that the limit of detection of the PCR method proposed might differ when applied to food samples on account of the processing they underwent. Moreover commercial samples different from those tested might need a specific extraction methods to obtain a pure DNA extract.

The availability of the duplex PCR method constituted the basis for the development of a PNA array device which allowed to simultaneously detect the presence of DNA from hazelnut and peanut, two ingredients which are possible source of hidden allergens in food products. The results here presented show that this combined approach can constitute a fast and reliable method for the indirect unambiguous determination of hazelnut and peanut in foodstuffs. Furthermore, when compared to conventional electrophoresis methods, this platform has the advantage to perform in one step the simultaneous detection of the presence of target organisms (i.e. hazelnut and peanut) and to confirm the identity of the amplified products via specific sequence recognition. On account of its sensitivity the method proposed is particularly suitable for detecting hidden allergens in food. The model here proposed can be easily further extended with new target DNA sequences and new PNA probes, in order to build up a complete array device for the detection of many potential sources of food allergens in foodstuffs.

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Chapter 6

Unconventional method based on circular dichroism to detect peanut DNA in food by means of a PNA probe and a cyanine dye

An innovative and unconventional method based on circular dichroism for the identification of peanut DNA in food was developed. The specific DNA target sequence can be detected after PCR amplification at the nanomolar level by using an achiral PNA probe complementary to the amplified tract of the peanut Ara h 2 gene, and the combined use of an achiral 3,3'-diethylthiadicarbocyanine dye ($DiSC_2(5)$). The optimized method was applied to the identification and quantification of DNA extracted and amplified by PCR from peanuts and from peanut-containing foods.

Stefano Sforza, Elena Scaravelli, Roberto Corradini, Rosangela Marchelli
"Unconventional method based on circular dicroism to detect peanut DNA in food by means of a PNA probe and a cyanine dye". Chirality, 2005; 17:1-7

Introduction

Peanuts are one of the most common causes of severe allergic reactions to foods even at minimal doses (100 μg of peanut proteins) (Hourihane et al., 1997a, 1997b), leading to life-threatening anaphylaxis and even death (Sampson et al., 1992; Yunginger et al., 1988). Avoidance of peanut-containing foods can be sometimes difficult for allergic patients because of mislabelling of the products, recycling of peanut-containing foods, or cross-contamination during processing. In order to protect the consumer, detection methods are required which specifically detect the presence of hidden allergens in a wide variety of food items.

Enzyme linked immunosorbent assays (ELISA) (Besler, 2001) are currently the most common methods to determine allergenic proteins in food products; however industrial processes often involve heat treatments, which may denature food proteins (Klein et al., 1984), thus modifying the protein structure and so interfering with their detection. In this case, an alternative is represented by the detection of the specific DNA of a determined species. DNA is less modified under heat and pressure processing and therefore it can provide a food index for the presence of hidden allergens in foods (Hird et al., 2003). The DNA extracted from food must be amplified by using PCR (Polymerase Chain Reaction). The presence of the amplified DNA product, which can be easily verified by gel electrophoresis, is taken as a proof of the identity of the specific DNA, although this is not always sufficient to get a definitive confirmation, since aspecific amplifications can often generate false positives, making necessary further confirmatory tests (Holzhauser et al., 2002; Borst et al., 2004; Prakoso et al., 2003).

One of the most promising approaches to confirm the DNA sequences after PCR amplification makes use of peptide nucleic acids (PNAs), oligonucleotide analogues with a pseudopeptide backbone (Nielsen, 2004), which bind to complementary DNA sequences through standard Watson-Crick hydrogen bonds and show a higher affinity for DNA than DNA itself, due to the absence of electrostatic repulsions between negative charges typical of DNA-DNA duplexes. Moreover, PNA-DNA duplexes are also characterized by a very high sequence specificity (Egholm et al., 1993). An HPLC method has been recently developed by our group for identifying specific DNA sequences by means of PNA probes (Lesignoli et al., 2001) and its use for the confirmation of the presence of hazelnut DNA previously amplified by PCR has also been reported (Germini et al., 2005).

In 1999 Armitage et al. reported that hybridization of PNA to DNA sequences can be readily visualized by using 3,3'-diethylthiadicarbocyanine dye ($DiSC_2(5)$, Figure 1), which strongly aggregates to PNA-DNA duplexes changing its absorbtion properties: the solution containing the dye alone in its prevailing monomeric state has a typical blue color, whereas upon aggregation to

the PNA-DNA duplex, the solution becomes purple, due to the appearance of a characteristic band at 540 nm (Smith et al., 1999).

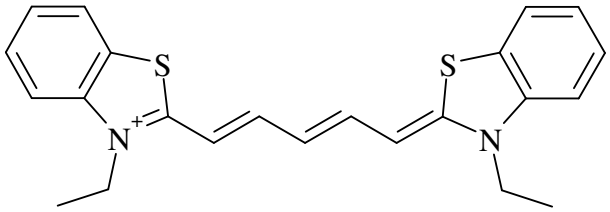

Figure 1: Structure of DiSC2(5) dye.

However, although outstanding results have been obtained for the detection of point mutations related to genetic diseases in humans (Wilhelmsson et al., 2002; Komiyama et al., 2003), several problems hamper its widespread use. First, the $DiSC_2(5)$ dye has a strong tendency to aggregate also on free PNAs, often generating confusing results. Moreover, the colorimetric detection has low sensitivity, requiring a DNA concentration in the low micromolar range, which is not easily obtained.

In this work we investigated the possibility to use of the $DiSC_2(5)$ dye and a PNA probe in order to confirm DNA sequences after PCR amplification by means of circular dichroism spectroscopy.

This technique has never been viewed before for this purpose. The rationale derives from the well known fact that, upon aggregation on PNA-DNA duplexes, not only a diagnostic absorbance band at 540 nm wavelength appears, but also a strong CD signal is generated in the same region, due to the asymmetric conformation of the $DiSC_2(5)$ dye aggregate induced by the PNA-DNA double helix. Moreover the right-handedness of the PNA-DNA helix also induces a right-handed helical arrangement of the dye aggregate, thus generating a strong exciton coupling effect which defines the shape of the CD band (Smith et al., 1999). The use of circular dichroism can overcome the limitations of the absorbance measurements, since it gives a positive signal only in the presence of chiral compounds: the $DiSC_2(5)$ dye, which is achiral, aggregates on the PNA-DNA duplex and on single strand PNA, but only the first one is a chiral complex, since the DNA is chiral but the PNA is not. Moreover, the strong intensity of the generated CD band may allow for a sensitive detection of DNA even at very low concentrations.

The DNA target of this work is an internal sequence of the peanut Ara h 2 gene (GenBank acc. number L77197) which codifies for one of the major allergen in peanuts. DNA detection, after amplification by Polymerase Chain Reaction (PCR), is based on the use of a complementary 15-mer PNA probe and the $DiSC_2(5)$ dye. The appearance of the CD signal in the 500-600 nm region with a right handed exciton coupling will confirm the presence and the identity of the amplified DNA. This method will be applied both to model systems and to foods.

Materials and methods

PNA design

The PNA sequence was designed in order to minimize any secondary structure which would result in a loss of hybridization efficiency, using the online available program Mfold (version 3.1; Zuker, 2003). The sequence specificity of the probe was first evaluated using the BLAST homology search system from DDBJ (http://www.ddbj.nig.ac.jp/E-mail/homology.html) (and then it was checked in order to avoid hybridization with any other non-target region among other amplified products.) The sequence chosen for the analyses is H-CGATCAACACATTCA-NH_2.

PNA synthesis

The PNA H-CGATCAACACATTCA-NH_2 was synthesised by solid-phase synthesis on a ABI 433A Peptide Synthesizer (Applied Biosystems) with the Boc strategy and HATU/DIEA coupling, as described previously (Lesignoli et al., 2001; Germini et al., 2005). Swelling, downloading and cleavage of the PNA from the resin were done manually. Two 2-(2-aminoethoxy)ethoxyacetic acid linkers were added at the N- terminus of the PNA for its eventual linking to solid surfaces (i.e. microarrays). The crude product was cleaved from the resin by using a 2:6:1:1 TFMSA/TFA/thioanisole/m-cresol mixture, precipitated by Et_2O and purified by reversed phase HPLC using a Phenomenex C18 peptide column (3 μm, 250 mm × 10 mm) with a binary gradient (flow rate: 4mL/min); eluent A: water /TFA = 100 : 0.2; eluent B: water/acetonitrile/TFA = 60 : 40 : 0.2; detector UV (260 nm). The purified product was characterized by ESI mass spectrometry (Micromass ZMD, positive ion mode): calcd. MW 4305 Da, calcd m/z: 862.0 (MH_5^{5+}), 718.5 (MH_6^{6+}), 616.0 (MH_7^{7+}) found m/z 861.9, 718.5, 615.9.

Oligonucleotide sequences

The oligonucleotide fully complementary to the PNA synthesized (5'-TGAATGTGTTGATCG-3'), to be used in the model experiments, and a non complementary oligonucleotide (5'-GAGCATTGAAATCAT-3') to be used as control in the same experiments, were purchased from Thermo Electron (Waltham, MA, USA).

Food samples

Unroasted peanuts without shell were provided by an italian food factory.
Muesli snacks with cereals and milk chocolate were commercially available. Declared content: Milk chocolate (21 %) sugar syrup, extruded cereal (wheat flour, rice, maiz malt, sugar and salt) toasted oat flakes, toasted peanut bits (7 %), brown sugar, hydrogenated vegetable oil, corn flakes (maize, sugar, salt, malt) whole condensed milk, toasted wheat flakes, toasted almond bits (1 %), honey (1 %), malt, emulsifying agent: mono and diglycerides of fatty acids; salt, flavours. Average cereal contents: 36 %.
Chocolate wafers were commercially available. Declared content: Cream filling 76 % (filling with cocoa 38 %: sweetener maltitol, vegetable fats, cocoa 11 %, whole milk powder, rice-starch, flavours; filling with vanilla flavour 38 %: vegetable fats, sweetener maltitol, whole milk powder, rice-starch, lecithin emulsifier agents, flavours), wheat flour, potato starch, vegetable oils, raising agents (sodium hydrogen carbonate), lecithin emulsifier agents, salt.

DNA extraction

DNA extraction was performed using the commercial kit Wizard® Plus Minipreps DNA purification system (Promega, Madison, WI, USA) opportunely modified as described in a previous work (Germini et al., 2005). The modifications can be summarized as follows: once the DNA was extracted and purified following the instructions provided by the commercial kit, it underwent a further purification step by addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and two volumes of cold 95 % ethanol, followed by incubation at –70 °C for 30 minutes. After precipitation the DNA was pelleted by centrifugation at 12000 rpm for 15 minutes at 4 °C. The DNA pellet was washed with 80 % ethanol and incubated at room temperature for 10 minutes, then centrifuged for 5 minutes at 12000 rpm; the DNA pellet was finally dried and then dissolved in 100 µl of water.

Polymerase chain reaction (PCR)

In order to develop a valid PCR method for the detection of specific DNA sequences coding for peanut allergens, several primer pairs were designed on known sequences obtained from on line available databases. The target sequence was the Ara h 2, one of the major allergens in peanut (GenBank acc. number L77197); the specificity of the possible PCR was firstly evaluated by aligning the designed amplicon with the available sequences using the database DDBJ Homology

search system and then tested by PCR. The target sequence was a 201 bp region identified by the primers Ara h 2_455 (5'-GGCGGCAGAGACAGATACTA-3') and Ara h 2_655 (5'-GACAACGCCATAAAAGCACTC-3').

Polymerase chain reactions were performed in a final volume of 100 µl. All the reagents were supplied by Euroclone (Wheterby, UK) and the primers purchased from Thermo Electron (Waltham, MA, USA).

The DNA extracted from peanuts underwent a double amplification: the first step was used to amplify the target sequences, while the second step was an asymmetric PCR used to selectively amplify the target DNA single strands to be hybridized with PNA, by using the double-stranded DNA obtained from the first PCR as the template to produce single-stranded DNA. The reagent concentrations in the first PCR were: 1x reaction buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 0.1 u/µl of hot start Blue Taq, 150 ng of extracted peanut DNA. For the second step (the asymmetric PCR), the reagent concentrations were the same, except the primer concentrations brought to 0.2 µM for the primer copying the non target DNA strand, and 2.0 µM for the primer copying the target DNA strand. Both PCR procedures were carried out in a PCR-sprint thermal cycler (Thermo Hybaid) using the following conditions: 1 cycle of DNA denaturation and Blue Taq activation at 95 °C for 5 minutes; 40 cycles consisting of DNA denaturation at 95 °C for 50 seconds, primer annealing at 60 °C for 50 seconds and elongation at 72 °C for 50 seconds; one step of final elongation at 72 °C for 5 minutes.

The DNA extracted from commercially available cereal snacks and chocolate wafers directly underwent to asymmetric PCR. In order to avoid false negative results generated by the residual presence of eventual PCR inhibitors, the extracted genomic DNA was diluted 20 times and only 4 µL of the diluted solution were added to the mastermix. All other reagent concentrations and conditions for the asymmetric PCR were the same above reported.

DNA-PNA hybridization

DNA-PNA hybridization tests were performed in a final volume of 2 mL: phosphate buffer (NaCl 100 mM, $NaH_2PO_4 \cdot H_2O$ 10 mM, EDTA 0,1 mM; pH 7), PNA (300 nM), DNA and water to 2 mL volume. The DNA used in the model experiments is a synthetic 15mer oligonucleotide complementary to the PNA used at different concentrations (from 1 to 300 nM). The DNA in the experiments making use of real food samples was the product of an asymmetric PCR of unknown

concentration (100 µL used). All samples were previously hybridized at 95 °C for 5' and then cooled to room temperature.

Addition of the $DiSC_2(5)$dye

$DiSC_2(5)$ dye was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of $DiSC_2(5)$ in methanol (500 µM) was added to each sample up to a final concentration of 10 µM in a total volume of 2 mL at room temperature; samples were then immediately analyzed in order to record the CD spectra.

Spectral measurements

CD spectra were recorded on a JASCO J-715 spectropolarimeter equipped with a Peltier thermostate (measurements done at 20°C). Acquisition range 400-700 nm, resolution 1 nm, accumulation 1, band width 1.0 nm, response 1 seconds, scan speed 100 nm/minutes. All spectra were treated with the noise reduction software included in the program J-700 for Windows Standard Analysis, version 1.33.00.

Results and discussion

The experimental design of the approach here proposed is represented schematically in Figure 2.

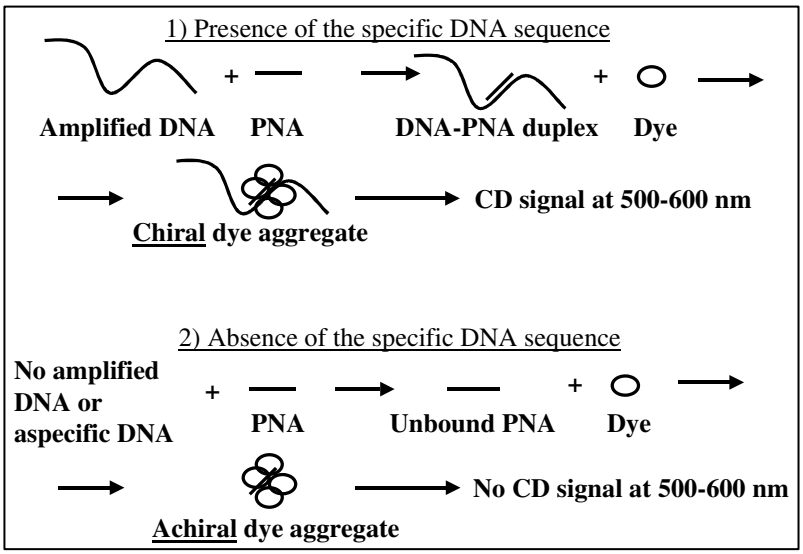

Figure 2: The rationale of the proposed method.
1) If the correct DNA sequence is amplified, the PNA will form a specific PNA-DNA duplex. Aggregation of the dye on this chiral duplex will generate a CD signal at 500-600 nm due to the induced chirality in the dye aggregate.
2) If no amplification occurs or if a wrong DNA sequence is aspecifically amplified, the PNA will not bind the DNA. The dye will nevertheless aggregate on the free PNA, but being the PNA achiral and the dye achiral, no CD signal will be generated at 500-600 nm.

As a model system to test our hypothesis, we used a 15-mer PNA designed to be complementary to a sequence contained in the Ara h 2 gene (the sequences are given in the experimental section), a gene which codes for the major peanut allergen.

In order to assess if a viable CD signal could be obtained by the dye aggregation onto the PNA-DNA duplex present at a low concentration and in order to define the best working concentration of the dye, we mixed the PNA strand (300 nM) with its complementary synthetic oligonucleotide (30 nM) in a phosphate buffer (pH = 7) and we added the $DiSC_2(5)$ dye to the solutions in concentrations ranging from 1 to 30 μM. The concentration of the complementary oligonucleotide was kept very low in order to simulate a likely DNA concentration of a PCR product. A well defined CD spectrum was recorded starting from a 5 μM dye concentration and the CD band appeared to level out at 10 μM, since addition of 20 or 30 μM $DiSC_2(5)$ dye did not increase the band intensity (data not shown). This simple experiment demonstrated that a very small amount of DNA can be detected by circular dichroism using PNA probes in connection with the $DiSC_2(5)$ dye and, according to the data obtained, a 10 μM concentration of the $DiSC_2(5)$ dye was chosen as optimal working condition.

In order to test the specificity of the sequence recognition, the 15-mer PNA was also mixed with a non complementary oligonucleotide in a phosphate buffer (pH = 7) and $DiSC_2(5)$ dye (10 μM) was added to the solution. The CD spectra of the PNA (300 nM) mixed with the fully complementary oligonucleotide (30 nM) and with a non complementary oligonucleotide (30 nM), both added of $DiSC_2(5)$ dye (10 μM), are reported in Figure 3. A CD spectrum of the solution containing only the dye and the complementary oligonucleotide, without any PNA, is also reported.The appearance of the CD band was clearly related to the presence of the specific PNA-DNA duplex, since in all cases when the duplex was not present (PNA with non complementary oligonucleotide or oligonucleotide alone), the signal was not significantly different form the baseline noise. It should be underlined that, although in the case of PNA mixed with the non complementary oligonucleotide no CD signal was detected, consistently with the likely absence of any PNA-DNA duplex, the solution nevertheless strongly absorbed at 540 nm and therefore became purple, since the dye strongly aggregated on the free PNA. This aspecific aggregation, although very disturbing in the case of absorbance measurements, is totally “invisible” in circular dichroism spectrophotometry, no chiral species being involved. Actually, since a fraction of the dye aggregates on the free PNA, this could result in a decrease of the available dye able to aggregate on the PNA-DNA duplex and could ultimately lead to a decrease of the potential CD signal, but the fact that the CD signal did not increase from 10 to 30 μM dye concentration confirmed that even at 10 μM concentration, being

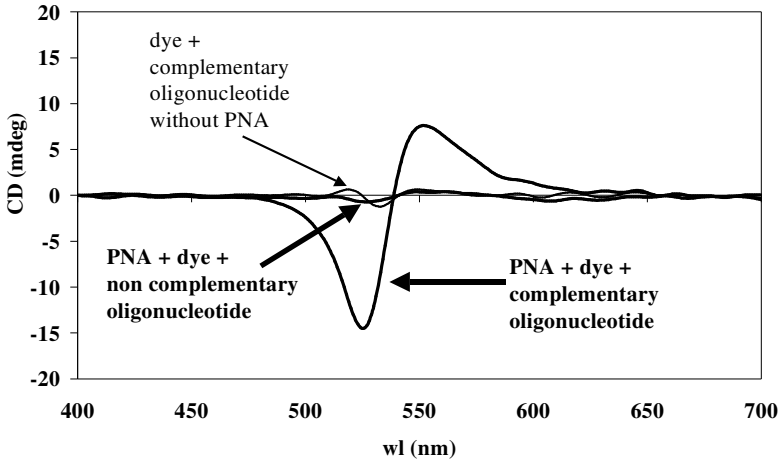

Figure 3: CD spectra obtained by adding the DiSC2(5) dye (10 μM) to:

i) a solution containing the 15-mer PNA complementary to a tract of the Ara h 2 gene (300 nM) and its complementary oligonucleotide (5'-TGAATGTGTTGATCG-3', 30 nM);

ii) a solution containing the same PNA and a non complementary oligonucleotide (5'- GAGCATTGAAATCAT-3', 30 nM)

iii) a solution containing the complementary oligonucleotide alone. (5'-TGAATGTGTTGATCG-3', 30 nM).

the dye present in large excess if compared to the PNA and to the DNA strands (and therefore also if compared to the PNA-DNA duplex), the fraction aggregated on the free PNA did not affect the intensity of the CD signal.

The absence of significant CD signals in the presence of the oligonucleotide and the dye is also of particular importance, since, being the DNA a chiral molecule, if the dye could interact with the single stranded DNA it may result in potentially interfering CD bands. In order to definitively rule out an interaction of the single stranded DNA with the dye, we have also added the non complementary oligonucleotide to the solution which contained the PNA, the complementary DNA and the dye, and we observed that the intensity and the shape of the CD spectrum were not influenced by this addition (data not shown). It should be underlined that ruling out an interaction of the dye with the single stranded DNA is of a particular importance in a real application with a DNA coming from a PCR amplification, where a short target DNA sequence is embedded in a very long DNA tract.

A further set of experiments was then performed in order to test the correlation between the oligonucleotide concentration and the CD signal, by mixing different concentrations of the oligonucleotide (1, 5, 10, 50, 100, 300 and 500 nM) with the complementary PNA (300 nM) and with the $DiSC_2(5)$ dye (10 μM). The spectra are reported in Figures 4 (from 1 to 100 nM oligonucleotide concentration) and 5 (from 50 to 500 nM oligonucleotide concentration).

A plot of the CD signal against the oligonucleotide concentration could be obtained by using the total CD signal (CD_{Tot}) defined as $CD_{Tot} = CD_{555nm} - CD_{525nm}$, where CD_{555nm} is the intensity of the CD signal at 555 nm (positive) and CD_{525nm} is the intensity of the CD signal at 525 nm (negative): the formula gives the sum of the absolute intensities of the two CD bands generated by the exciton effect. Every experiment at every different concentration was repeated three times and the average results were used in the plot. The plots are reported as inserts in Figures 4 and 5.

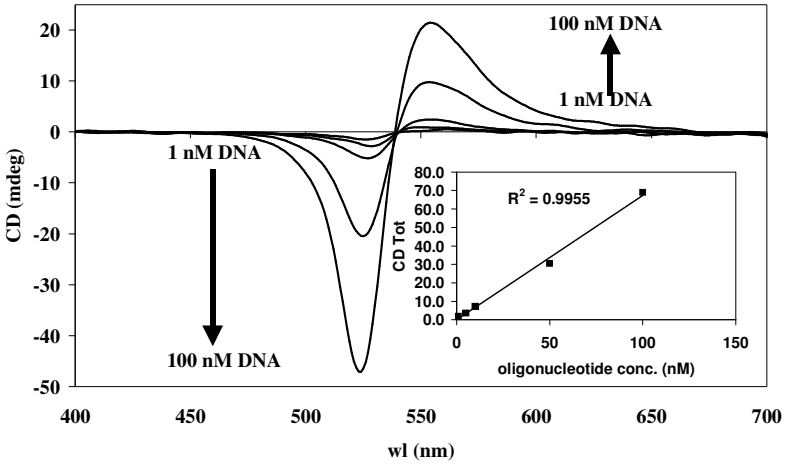

Figure 4: Increase of the CD signal according to the oligonucleotide concentration in solutions containing the 15-mer PNA complementary to a tract of the Ara h 2 gene (300 nM), the complementary oligonucleotide (5'-TGAATGTGTTGATCG-3', concentrations: 1, 5, 10, 50, 100 nM) and the DiSC2(5) dye (10 μM). The plot of the total CD signal against the oligonucleotide concentration is shown in the insert with the corresponding linear regression.

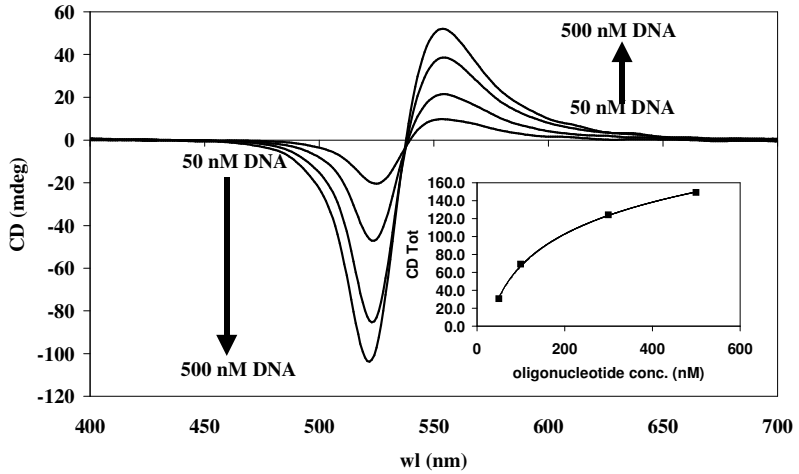

Figure 5: Increase of the CD signal according to the oligonucleotide concentration in solutions containing the 15-mer PNA complementary to a tract of the Ara h 2 gene (300 nM), the complementary oligonucleotide (5'-TGAATGTGTTGATCG-3', concentrations: 50, 100, 300 and 500 nM) and the DiSC2(5) dye (10 μM). The plot of the total CD signal against the oligonucleotide concentration is shown in the insert with the corresponding regression.

The value recorded at a 1 nM concentration of the oligonucleotide was not significantly different from the value recorded with the non complementary oligonucleotide: as a consequence, the limit of detection of this method can be approximately estimated to be 5 nM DNA concentration. It is immediately evident that the response is related to the oligonucleotide concentration and it is also linear up to 100 nM (Figure 4), whereas the signal has a tendency to level off at higher concentrations (Figure 5). Therefore, in order to have a linear response, it appears that the PNA has to be present in excess as compared to the DNA strand. The linearity of response has important consequences as far as DNA quantification is concerned. If we assume that the intensity of the CD signal only depends from the PNA-DNA duplex concentration, then it should not be strongly influenced if the target DNA sequence is part of a much longer DNA sequence, as it happens in a PCR product. Therefore, we can use the linear regression obtained with the complementary oligonucleotide to "calibrate" the method, making an accurate quantification of the amount of DNA after the PCR amplification feasible.

On the base of the promising results obtained in the model system with the complementary oligonucleotide, the method was tested in real samples, by using PCR products instead of a synthetic oligonucleotide.

Unroasted peanuts were used as the first sample: the DNA was extracted and a 201 base pair region of the Ara h 2 gene was first amplified by a standard PCR procedure. Since a PCR yields double stranded DNA and given the fact that PNA can hybridize only to single stranded DNA, a further PCR was then performed by using different concentrations of the two primers (asymmetric PCR), in order to have a certain amount of single stranded DNA which could be recognized and bound by the PNA. The details of DNA amplification starting from peanuts are reported in the experimental section. After the asymmetric amplification, 100 μL of the mixture containing the amplified DNA was mixed with the PNA (300 nM) and the $DiSC_2(5)$ dye (10 μM). A strong signal was obtained, as reported in figure 6, allowing to confirm the presence of a DNA containing the target sequence, i.e. a part of the Ara h 2 gene, as it was expected.

A more interesting experiment was then performed by trying to assess the presence of peanut DNA in cereal snacks containing peanuts (peanut declared content: 7 %): the DNA was extracted and the region of the Ara h 2 gene directly amplified by using only asymmetric PCR, without preamplifying the DNA by standard PCR. In order to test a negative sample, the DNA was also extracted and amplified by asymmetric PCR also from a chocolate wafer not containing peanuts. After adding the PNA probe and the $DiSC_2(5)$ dye, the CD spectra were recorded and are also reported in Figure 6. This experiment consistently gave positive signals only in the presence of the peanut-containing sample.

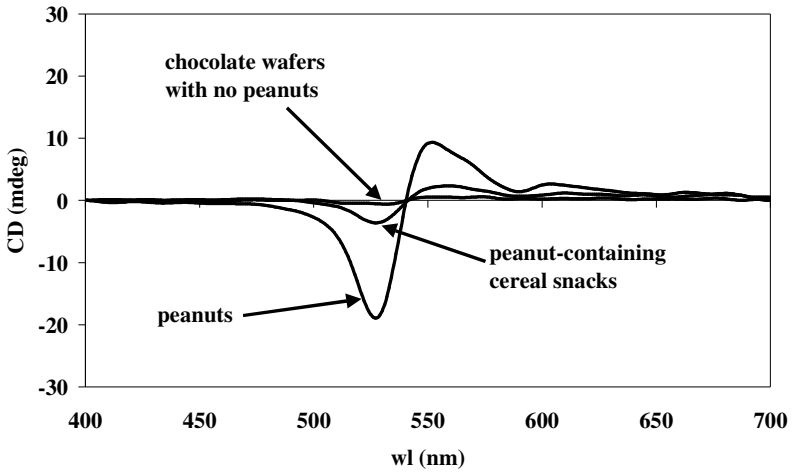

Figure 6: CD spectra obtained by adding the DiSC2(5) dye (10 μM) to:
i) a solution containing the 15-mer PNA complementary to a tract of the Ara h 2 gene (300 nM) and the DNA extracted and amplified from unroasted peanuts;
ii) a solution containing the 15-mer PNA complementary to a tract of the Ara h 2 gene (300 nM) and the DNA extracted and amplified from peanut-containing (7%) cereal snacks;
iii) a solution containing the 15-mer PNA complementary to a tract of the Ara h 2 gene (300 nM) and the DNA extracted and amplified from chocolate wafers with no peanuts.

The linear regression obtained as reported above was used to quantify the single stranded DNA present in the two positive cases, by assuming that the aggregation of the dye is influenced only by the PNA-DNA duplex. We obtained a value of 41 nM in the case of DNA coming from 100 % peanuts (corresponding to an absolute amount of single strand DNA of 82 pmol) and a value of 9 nM for the DNA coming from the cereal snacks (corresponding to an absolute amount of single strand DNA of 18 pmol).

It should also be noted, as a final remark, that the extreme sensitivity of the method requires the use of a very specific PNA probe. Whenever in the post-PCR mixture an amplified DNA is present which does not correspond to the target sequence, in order to avoid false positives, it is absolutely mandatory that the PNA probe bears less than 4-5 bases complementary to any tract along the overall DNA sequence. In fact, preliminary experiments showed that a complementarity of 5 bases or more will give rise to PNA-DNA duplexes stable enough to elicit $DiSC_2(5)$ dye aggregation and therefore the appearance of CD signals which, in that case, are false positives.

Conclusions

We report for the first time a method based on circular dichroism for the confirmation, after PCR amplification, of a specific DNA sequence in foods by using a PNA probe and the diethylthiadicarbocyanine dye. The method is based on the appearance of a diagnostic absorption

band at 540 nm, due to the aggregation of the dye onto the PNA-DNA duplex, accompanied by the selective elicitation of a CD signal at the same wavelength due to the chirality of the dye aggregate induced by the right-handedness of the PNA-DNA duplex. The method appears to be very selective, since the free PNA/dye aggregate, which exists in solution, does not give rise to any CD signal, being achiral, and the dye does not aggregate on free DNA. The method was demonstrated to be very sensitive, allowing the confirmation of DNA down to a 5 nM concentration, and the response was shown to be linear in the 5-100 nM range.

Application to real food samples demonstrated that the method can be used for the confirmation of peanut DNA after PCR amplification also in complex food matrices, making this approach a specific and efficient way to confirm the presence of hidden allergens in food.

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Chapter 7

Light up probes in real-time PCR for peanut detection

The possible application of a Peptide Nucleic Acid (PNA) based probe, a so called Light Up probe, in real-time PCR detection of peanut has been investigated. The Light-up probe is a linear probe in which a PNA probe is conjugated the thiazole orange (TO) as fluorophore,.
Preliminary results showed that this innovative method is capable to detect peanut DNA with sensitivity and efficiency that are comparable to that of current real-time PCR detection systems.
Although further studies are still needed to assess and improve the specificity of the new method, it might have the potential to develop into a future methodology that can be employed to detect traces of peanut in food.

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Arjon J. van Hengel and Rosangela Marchelli

Introduction

The availability of accurate and sensitive detection methods for hidden allergens in food products is crucial for either the food industry to control and ensure the correct labelling of their products and for government regulatory agencies in order to enforce legislation (European Parliament and Council, 2000; 2007). Peanut is included in the list of food allergens that have to be declared on the labels and it is known as one of the most allergenic (Bock and Atkins, 1990; Sampson et al., 1992); even the intake of minimal doses (e.g. 200 µg) can be sufficient to trigger an allergic reaction (Tariq et al., 1996; Wensing et al., 2002).

The development of any detection method relies on the identification of a target analyte which can be the allergenic protein or the DNA sequence coding for a specific protein. DNA detection methods are based on the Polymerase Chain Reaction. They are currently used for the detection of microbial pathogens (Malorny et al., 2003), genetically modified organisms in food products (Anklam et al., 2002; Holst-Jensen et al., 2003; Germini et al., 2004; Germini et al., 2005) and more recently also for food allergens in foodstuffs (Poms and Anklam, 2004).

PCR is an enzymatic reaction that consists in 25-45 cycles which allow the amplification of a DNA target sequence up to a level that can be revealed. Real-time PCR is based on fluorescence detection: the presence of the amplified product is revealed by detection of a fluorescent signal which is monitored while the amplification occurs. The use of fluorescent dyes allows the amplification and detection steps of the PCR to be combined but, according to the different dye chemistries applied, real-time PCR can be grouped in two main categories: *unspecific* and *specific detection*.

When the detection is *unspecific*, dyes intercalating any double stranded DNA (dsDNA) are employed. The first dye that established the proof-of-principle was the ethidium bromide (EtBr) (Higuchi et al., 1992, 1993). EtBr was added to the PCR reaction with continuous monitoring during the cycles; a plot of the increased fluorescence caused by EtBr binding the newly synthesised DNA against PCR cycles allowed calculating the amount of starting material. Several dyes are now commercially available and the most commonly used are SYBR Green ® I (Morrison et al., 1998; Karlsen et al., 1995) or SYBR Gold ®. Although these DNA-binding dyes are relatively inexpensive and can be incorporated into already optimized protocols, also significant disadvantages may be involved. The random binding of the dyes to any dsDNA results in non specific fluorescence and the specificity of the reaction, as with conventional PCR, is entirely determined by its primers.

On the contrary, the template *specific* analysis is based on the use of a fluorescent probe specifically designed per each PCR assay. The fluorescent signal is only generated if the amplicon-specific probe hybridizes to its complementary target.

The advantage of fluorogenic probes, over DNA binding dyes, is that specific hybridization between probe and target is required to generate the fluorescent signal. This means that non-specific amplifications due to mis-priming or primer-dimer artifacts do not generate signals. Another advantage of fluorogenic probes is the possibility to label each probe with a different, distinguishable reporter dye which allows the amplification and individual detection of distinct sequences in a single PCR reaction. The disadvantage of fluorogenic probes is that different probes must be synthesized to detect different sequences.

As listed in Table 1, probes can be grouped in linear and structured molecules (Bustin and Nolan, 2004). Linear probes have the advantage of a better binding efficiency because the absence of any secondary structured avoids competition with intramolecular hybridization. On the other hand, thermodynamic studies showed that structured probes result in a higher hybridization specificity and better recognition of mismatches (Bonnet et al., 1999).

Table 1: list of linear and structured probes used in real-time PCR. The mechanism of fluorescence is distinguished in presence of only one fluorophore (F) or one fluorophore and one quencher (F & Q) combined to get the signal emission.

Type	Probe	Fluorophore / Quencher	References
Linear Probes	ResonSense® and Angler®	F & Q	Lee et al., 2002
	Hybeacons ™	F	French et al., 2001, 2002
	Light up probes	F	Svanvik et al., 2000a, 2000b, 2001; Isacsson et al., 2000; Wolffs et al., 2001
	Hydrolysis (TaqMan®) Probes	F & Q	Holland et al., 1991; Lee et al., 1993 ; Livak et al., 1995
	Lanthanide Probes	F	Nurmi et al., 2000, 2002
	Hybridization Probes	F & Q	Pals et al., 2001; Aslanidis 1999
	Eclipse™	F & Q	Afonina et al.,2002
	Strand-displacement probes (Yin-Yang probes)	F & Q	Li et al., 2002; Shengqi et al., 2002
Structured Probes	Molecular Beacons	F & Q	Mhlanga et al., 2001; Vet et al., 2005; Kuhn et al., 2001, 2002; Totsingan et al., in press)
	Scorpions™	F & Q	Whitcombe et al., 1999; Solinas et al., 2001; Thelwell et al., 2000
	Cyclons™	F & Q	Jiang et al., 1999; Kandimalla 2000

Another fluorogenic probe characteristic is the mechanism of fluorescence generation: it is different among the different types of probes. They all are labelled with at least one fluorophore but in some cases they are equipped with a fluorescence donor-acceptor pair of dyes. Therefore the fluorescence detection technique could be based on the quenching of fluorescence by energy transfer from one fluorophore to another fluorophore or to a non-fluorescent molecule (Marras, 2006). This particular probe design is widely used for the TaqMan® probes: an acceptor dye, known as quencher, receives energy from the donor fluorophore and dissipates it by fluorescence resonance energy transfer (FRET). If the acceptor is a fluorophore, the transferred energy is emitted as fluorescence, characteristic for the acceptor fluorophore, whereas when the acceptor is not fluorescent, the absorbed energy is lost as heat without any fluorescent emission.

In this study the possible application of a light up probe in real-time PCR for peanut allergens detection has been investigated. The Light-up probe is a linear probe composed of only one fluorophore, the thiazole orange (TO), conjugated to a PNA sequence (Figure 1).

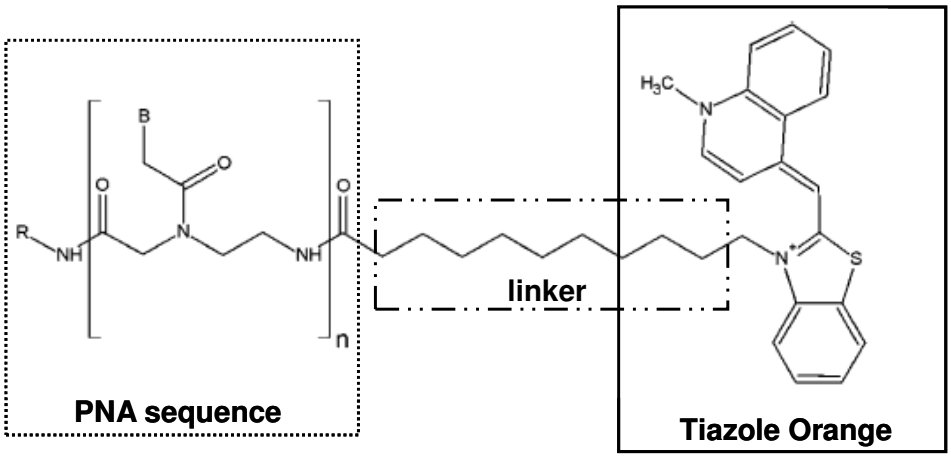

Figure 1: Structure of a light up probe

TO is a derivative of an asymmetric cyanine dye; in this type of dye, the two aromatic systems can rotate around the interconnecting methylene bond. The linker in the light up probe is flexible in order to allow the dye to interact with the target nucleic acids hybridized to the PNA sequence. Due to this interaction, the aromatic moieties becomes fixed in a co-planar geometry and the dye gives rise to a strong fluorescent signal which can be readily detected (Svanvik et al., 2000a, 2000b).

Materials and Methods

Light up probe (LUPeanut)

PNA H-CTC TTC TTG TCT-NH_2 was synthesized by an automated Fmoc-SPPS protocol (Solid Phase Peptide Synthesis) on a ABI 433A synthesizer, following the procedures provided by the company. The thiazole orange (TO) derivative (6-TO exanoic acid) was introduced by manual solid phase synthesis, by using a DIC (Diisopropylcarbodiimide) / DhBTOH (3-Hydroxy-1,2,3-benzotriazin-4(3H)-one) coupling protocol. The free PNA was cleaved from the resin by using a 9:1 mixture of TFA/m-cresol and precipitated by Et_2O. The residue was dissolved in water and purified by using a water pre-equilibrated Sep-pak® C18 cartridge. Coloured eluates obtained upon elution of H_2O/MeCN = 70/30 were collected and analyzed by HPLC and ESI-MS spectrometry. ESI calcd m/z: 1189 (MH_3^{3+}), 892 (MH_4^{4+}), 713.8 (MH_5^{5+}), 595 (MH_6^{6+}), found m/z: 1189, 892, 714, 595.
Molar absorptivity of the probe at 260 nm was estimated as the sum of the contributions from PNA and TO (Nygren et al., 1998): $\varepsilon_{260} = 11{,}700_1G + 26800_4C + 60200_7T + \varepsilon_{260}TO$ 7100 = 105 800 M^{-1} cm^{-1}. The concentration of the probe was determined by absorption measurements at room temperature (Ultraspect pro 3300, Amersham Biosciences, Biochrom Ltd, England).
The thermal melting temperature (Tm) of the probe–target complex in the PCR buffer (Qiagen GmbH, Germany) was estimated by determining the Tm for a probe–oligonucleotide complex (2 μM), by A260 measurements. The oligonucleotide (Sigma Genosys) had the sequence 5'AGA CAA GAA GAG 3'. The measurement of the melting temperature was carried out on the LS55 Luminescence Spectrometer (Perkin Elmer, USA).

Real-time PCR conditions with light up probe:

The amplicon sequence of PCR B (Scaravelli et al., *in press*), designed on the DNA sequence coding for the peanut allergen Ara h 3, was used for real-time amplification with the specifically designed light up probe LUPeanut.
To optimise the performance of the PCR reactions with the light up probe, the following parameters were tested: PCR mastermixes (TaqMan® Universal PCR Master Mix, Applied Biosystems, UK; HotStartTaq Master Mix Kit, Qiagen GmbH, Germany), LUPpeanut concentrations (0.1, 0.25, 0.5, 1 μM) and thermal cycle (Table 2).
The optimal assay was set as follows: primer concentration of 0.9 μM, probe concentration 1 μM, around 100 ng of template DNA, final volume of 25 μl, PCR mastermix HotStartTaq Master Mix

Table 2: list of thermal cycles tested. In bold the steps of data acquisition

Thermal cycle	DNA Denaturation	40 cycles			Melting curve		
		DNA denaturation	Primers annealing	Sequence elongation			
1	95C/10'	95C/15"	**55C/1'**	72C/30"	95C/15"	***35C/1'***	***95C/15"***
2	95C/10'	95C/15"	**60C/1'**	72C/30"	95C/15"	***35C/1'***	***95C/15"***
3	95C/10'	95C/15"	**60C/30"**	72C/30"	95C/15"	***35C/1'***	***95C/15"***
4	95C/10'	95C/15"	**65C/45"**	72C/30"	95C/15"	***35C/1'***	***95C/15"***
5	95C/10'	95C/15"	**65C/45"**	72C/30"	95C/15"	***35C/1'***	***95C/15"***
6	95C/10'	95C/15"	**75C/1'** + 60C/15"	72C/30"	95C/15"	***35C/1'***	***95C/15"***

Kit (Qiagen GmbH, Germany) and with the thermal cycling 2 as listed in Table 2. In each thermal cycle, a final step for analysing the melting curve with fluorescence monitoring was included (*dissociation curve*), with a temperature ramp spanning from 35 to 95 °C and a ramp rate of 2 % as suggested by the instrument manufacturer. Signal acquisition started at 35 °C and continued during the entire step.

In order to test the probe specificity, two non-peanut specific DNA sequences were amplified by adding their own specific DNA and the peanut specific light up probe. The two pairs of primers used were: AlfaFor 5'-AGAAACGGACTTGAGGAGACA-3' and AlfaRev 5'-AGCAGCAAGTCCAAGCCA-3'; DeltaFor 5'-TGTGAGCAACT- GAATGAGCTTAA-3' and DeltaRev 5'-AAACCCACAAGTCCTAGGCAA-3'.

All reactions were run on the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, UK). Fluorescence was monitored at the end of the annealing phase using 495 nm excitation and 518 nm emission (the 6-FAM channel of the instrument).

In each analysis and for each parameter tested, a positive (adding peanut DNA), negative (adding non peanut DNA) and non template (adding water) control were always included. All results were analyzed by setting the automatic baseline of the instrument and 0.30 as threshold (no passive reference).

The final Ct values used for quantitative calculation refer to the mean value of three replicates of amplification.

Real-time PCR using Taqman probes

The set of primers and probe (TaqMan®) for the amplicon sequence PCR B (Scaravelli et al., *in press*), designed on the DNA sequence coding for the peanut allergen Ara h 3, was used for real-time amplification according to the optimised condition previously described. In each 96 wells plate, a positive (with peanut DNA) and a non template (adding water instead of DNA) control were always included in the analysis.

Results and discussion

Many probes (Table 1) have been studied in order to improve the PCR performances, especially when traces of a certain DNA target need to be detected. Some commercial (Tepnel, UK; Congen / R-Biopharm, Germany) or published methods (Hird et al., 2003; Stephan and Vieths, 2004, Scaravelli et al., *in press*) for peanut allergens DNA detection are already available. Most of them are based on conventional PCR coupled with gel electrophoresis or, in the case of real-time PCR, they mainly apply the TaqMan® chemistry. With the aim of achieving a better sensitivity in an already developed real-time PCR protocol, a study on the application of PNA as fluorescence-monitoring systems for DNA amplification is reported in this work. Light up probes combine the advantages of PNA probes with the fluorescence properties of TO: hybridization to DNA with high sequence specificity and with up to 50-fold increase in fluorescence (Svanvik et al., 2000a). Moreover these hybridization probes are provided with a single reporter dye (TO), which is sufficient to monitor the increase in fluorescence intensity, instead of measuring the change in the fluorescence intensity distribution necessary when using energy transfer probes.

A 12 mer light up probe targeting a 105 bp fragment of the Ara h 3 gene amplified by the primers of the PCR B assay (Scaravelli et al., *in press*), has been specifically designed and successfully synthesized as confirmed by the ESI-MS spectrum (Figure 2).

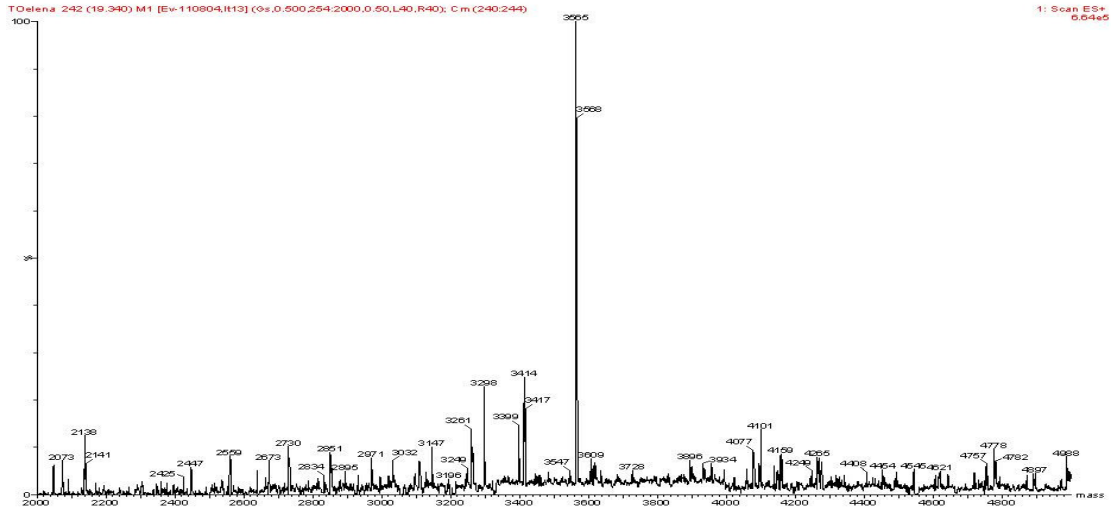

Figure 2: Spectrum from ESI-MS analysis of the LUPeanut (MW calculated = 3564.2; MW found = 3565, $M+H^+$)

Thanks to the particular hybridization properties of PNA, the interaction of light-up probes with target nucleic acid depends on the probe sequence. In this case a pirimidine reach sequence has been chosen because it could form a PNA2–DNA triplex (Egholm et al., 1992; Kim et al., 1993).

Homopiridine sequences are known to be particularly stable and exhibiting very low fluorescence as free probe (Svanvik et al., 2000a).

When designing the light up probe, the sequence specificity was one of the main requirements to fulfil. It was difficult to predict the correct Tm because many factors could influence the theoretical calculation such as the possible formation of triplex (PNA2-DNA) with a pirimidine rich sequence, and an increased stability due to the interaction of TO linked to the PNA probe with the DNA strand, as previously shown. The theorical Tm of the LUPeanut/DNA duplex without TO was calculated at 48 °C but the measured Tm was 58 °C.

According to recent studies, the light up probe should bind the DNA at the annealing step during the PCR reaction and dissociate during the extension phase without interfering with the reaction itself (Kubista et al., 2001).

In order to test the influence of LUPeanut on the PCR reaction, the amplicon sequence PCR B was amplified in presence of different concentrations of light up probe (0.1, 0.25, 0.5, 1μM) and the fluorescence enhancement during the annealing step (thermal cycle 2 from Table 2) was monitored. Figure 3 shows the amplification plot obtained by this amplification series. Thanks to the probe design, LUPeanut did not inhibit DNA amplification, as might have been expected from PCR clamping studies. But, despite previous findings (Kubista et al., 2001), the probe concentration

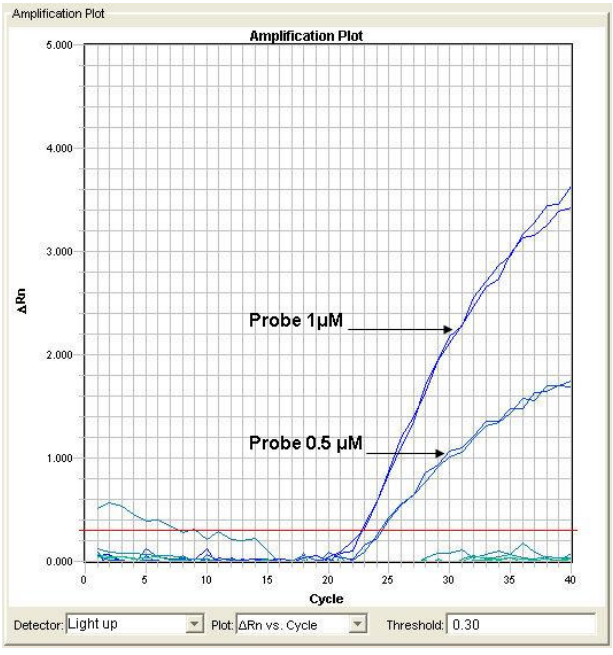

Figure 3: amplification plot obtained with different LUPeanut concentration (0.1, 0.25, 0.5, 1 μM)

seems to affect PCR efficiency. Probe concentrations equal or lower than 0.25 μM did not produce any fluorescent signal and the best result was obtained with the highest probe concentration (1 μM). The fluorescence signal was obtained by utilizing the HotStartTaq Master Mix Kit (Qiagen GmbH, Germany); the two mastermix reagents tested seemed to influence the PCR reaction in the presence

of the light up probe. The agarose gel (data not shown), confirmed an intense amplification lane at 105bp, only when using mastermix from Qiagen (Qiagen GmbH, Germany); on the contrary the mastermix from Applied Biosystem (Applied Biosystems, UK), normally used in the optimized condition of the PCR B assay, did not allow an efficient amplification. A possible explanation could be the different efficiency of the two Taq Polymerases activity in presence of a PNA probe in solution or the different components of the PCR buffers.

In order to check the specificity and the free probe fluorescence during the amplification process, the negative control and the non template control (NTC) were also tested for each probe concentration. No fluorescence signal resulted from both PCR reaction tested: in the presence of the specific PCR B pair of primers and a non peanut DNA extract (negative control), no aspecific amplified product was detected by the LUPeanut probe; moreover no fluorescence enhancement was detected when water was added to the mastermix instead of a DNA extract (NTC). These results highlighted the absence of primer-dimer artifacts, as also confirmed by gel electrophoresis, the specificity of the couple of primers of the PCR B assay and the ability of the probe not to give a high background if dsDNA is not synthesised during the PCR process.

Since the results obtained were comparable to the PCR B assay which is based on TaqMan® chemistry, the sensitivity of the new light up probe based assay has been tested by amplifying peanut DNA dilutions ranging from 1 pg to 100 ng. The Cycles Threshold (Ct: the cycle at which the fluorescence signal is higher than the background) collected were used to generate a linear curve, by plotting the Ct values against the logarithm of the amount of DNA (Figure 4).

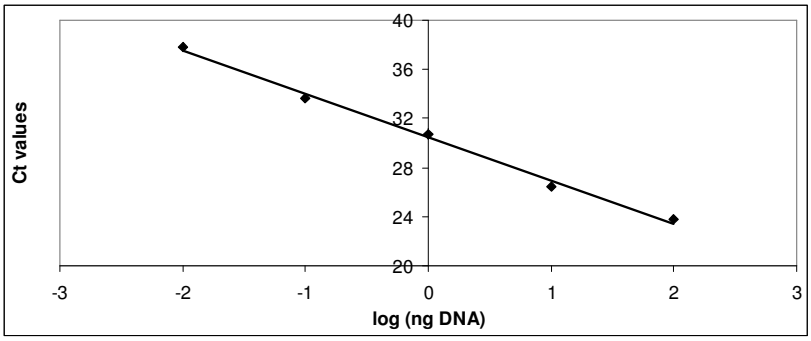

Figure 4: linear correlation obtained by plotting the Ct values against the logarithm of the DNA amount in the PCR reaction. Peanut DNA dilutions (0.001, 0.01, 0.1, 1, 10, 100 ng) were used for the PCR reactions.

The detection limit of the light-up probe was 10 pg DNA per reaction tube when three independent PCR reactions were carried out. According to our previous findings on the sensitivity of the PCR B assay using its specific TaqMan probe, PCR B can detect up to 2.5 pg of peanut DNA, which corresponds to less than one peanut genome equivalent (Scaravelli et al., *in press*). The fluorescence

efficiency of the light up probe in the present PCR condition allowed the detection of three peanut genome equivalent which is a value comparable to the already optimized method.

Studies in progress

The specificity of the LUPeanut method was cross checked also by amplifying two non peanut specific amplicons, as described in the Material and Methods section, in presence of the light up peanut specific probe. The amplification plot showed a fluorescence enhancement also when the sequences amplified were not the PCR B amplicon. From the fluorescent melting curve (*dissociation curve*), the Tm of the positive and false-positive samples were similar (Tm = 80°C) which could be explained by the high stability of the hybrids formed by the LUPeanut with DNA synthesised during the PCR process.

The first critical step in achieving high probe specificity is represented by the probe design. In this case the selection of the specific 12 mer sequence was carried out avoiding any complementarity with all the vegetable sequences present in the available database (http://www.ncbi.nlm.nih.gov/).

Another critical step, that has to be considered when aiming at the specificity from both primers and probe, is the thermal cycle and the point of signal acquisition. Different conditions and point of data acquisition during the thermal cycle were tested as listed in Table 2 (Material and Methods section) in order to destabilize and discriminate the non-fully complementary sequences. None of the parameters tested could significantly reduce the fluorescence signal coming from the false-positive samples. A possible explanation could be that the linker is too long and could also allow unspecific interactions of TO with other dsDNA sequences, without hybridization of the PNA sequence giving rise to a fluorescence detectable signal. Another reason for aspecificity would be the dependence of the fluorescence response from the concentration of the light up probe, which has been shown to decrease dramatically at a low (0.1 μM) concentration of the probe. Other experiments carried out in solution and analysed in a normal spectrofluorimeter showed that an excess of probe led to aspecific interactions with dsDNA (data not shown).This means that the concentration range in which the probe can still discriminate different dsDNA sequences has to be investigateed in detail. Moreover, from experiments with complementary and non-complementary oligonucleotides, there was no evidence for the formation a triplex since no hysteresis was observed (data not shown).

Another problem is the long term stability of the probe. The purified product needs to be stocked dried and redissolved in small aliquots when required to be used.

Conclusions

The preliminary results obtained indicate that sensitivity and efficiency comparable to that of current real-time detection systems can be obtained without extensive optimization. However, a deeper investigation on the aspecific hybridization of the PNA sequence or indiscriminate binding of the TO to dsDNA is necessary for the critical evaluation of LUPeanut assay effectiveness to be compared to other probe systems (e.g. TaqMan®).

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Concluding remarks

A major concern in the protection of allergic consumers is the availability of detection methods that are able to identify the presence of trace amounts of allergenic ingredients in food products. In this thesis multiple DNA based methods for the detection of peanut, which is considered one of the major food allergens, have been developed. Three real-time PCR methods have been developed and optimized all targeting a specific region of the peanut allergen Ara h 3. Cookie and chocolate model matrices were developed and used as "standard test materials". This allowed the application of the method for semi-quantitative purposes in a range between 10 and 10000 mg kg-1. The sensitivity that can be achieved with the developed real-time PCR methods was found be in line with clinical thresholds. A comparative study employing real-time PCR and ELISA revealed a similar performance for both detection methods. Furthermore, ELISA and real-time PCR were found to be affected in a similar manner by heat treatments undergone by the food samples. Analysis of hundreds of market samples stressed that the availability of multiple and diverse detection methodologies is a key issue in food allergen detection since each technique can be differently affected by factors like the food matrix, the allergen targeted and the industrial processes employed.
A detailed knowledge of the performance as well as the advantages and disadvantages of each method contributes in the assessment of the reliability of protein and DNA based methods and supports the selection of the most suitable method.
The need of multiple and diverse detection methods constituted the basis for further studies employing innovative techniques. The development of a PNA array device allowed the simultaneous detection of DNA from hazelnut and peanut in food products. Moreover, the unconventional application of circular dichroism offered another sensitive method that can be used for the detection of peanut allergens in complex food matrices. New studies on the use of Light Up Probes in real-time PCR were initiated and might develop into future methodologies that can be employed to detect hidden allergens.
All the investigated techniques and their further applications contribute to the protection of allergic consumer and provide tools that are essential to reduce the risk of the accidental ingestion of hidden allergens.

Annex I

Annex I

Cookies

		ELISA				DISPSTICK	RT PCR			RT PCR-ELISA				RT PCR-DIPSTICK	ELISA- RT PCR-DIPSTICK	ingredients		"may contain"		present in factory		cocoa /choco content %
	sample no	0.7-10ppm	10-20 ppm	>20 ppm	pos / neg		0.7-10ppm	10-20 ppm	>20 ppm	0.7-10ppm	10-20 ppm	>20 ppm	QUALIT			PEA	NUT	PEA	NUT	PEA	NUT	
Germany	20	0.9			P									X								7.5
	33	7.9			P									X					x			
	40	1.0			P									X								
	43			>20ppm	P	P			568.0			X	X	X	X					x	x	
	44	3.4			P	P	POS						X	X	X				x			
	46			20.23	P	P			81.0			X	X	X	X			x	x			30
Belgium	184	2.5			P		3.6			X			X					x	x			12
	231			> 20ppm	P				37.0			X	X									5.6
	232	1.9			P									X								
	244	3.7			P	P	4.0			X			X	X	X			x	x			
	256			> 20ppm	P	P			368.0			X	X	X	X			x	x			15
The Netherlands	318			>20ppm	P	P	POS						X	X	X					x	x	
	367	2.2			P		3.0			X			X							x	x	
	391	1.2			P															x		
Austria																						
Czech Republic	319			>20ppm	P	P			20.8			X	X	X	X					x		?
	320			>20ppm	P	P			86.0			X	X	X	X			x				?
	325			>20ppm	P	P			86.4			X	X	X	X					x		34
	329	2.0			P	P														x		30
	331			>20ppm	P	P		12.3					X	X	X					x		30
	333	2.6			P	P	2.6			X			X	X	X							?
	339	6.7			P		POS						X									4.6
	343			> 20ppm	P	P			437.0			X	X	X	X			x	x			7.5
	344			> 20ppm	P	P			7677.0			X	X	X	X			x				7.5
	345			> 20ppm	P	P			222.0			X	X	X	X							20
	347		10.0		P	P		15.0			X		X	X	X			x	x			14
	349			> 20ppm	P	P			254.0			X	X	X	X							?
	352	4.3			P		4.4			X			X									?
	353	1.8			P		POS						X									
	354	2.3			P	P	POS						X	X	X					x		3.9
	355			> 20ppm	P	P			26.5			X	X	X	X							65
	358			> 20ppm	P				24.6			X	X					x				?
	359			> 20ppm	P	P			164.0			X	X	X	X			x				?
	360			> 20ppm	P	P		11.7					X	X	X			x				28
	364			> 20ppm	P	P			392.0			X	X	X	X			x	x			48.5
Poland	425	1.1			P									X								
	435			> 20ppm	P	P	5.8						X	X	X							
Hungary	442			>20ppm	P	P	POS						X	X	X			x	x			5
Slovakia	505			> 20ppm	P	P			41.0			X	X	X	X							?
	506		11.0		P	P		18.2					X	X	X							20
	507			> 20ppm	P	P			10966.0			X	X	X	X							20
	508			> 20ppm	P	P			320.6			X	X	X	X							24
	509			> 20ppm	P	P			1231.0			X	X	X	X							14
	510			> 20ppm	P	P			51.4			X	X	X	X							7
	511	3.2			P	P	5.8			X			X	X	X							14
	513	2.2			P									X								?
	514	2.3			P		3.5			X			X									
	517	1.2			P		8.8						X									25
	518			> 20ppm	P	P			86.6			X	X	X	X							24
	520			> 20ppm	P	P			629.0			X	X	X	X							1
	523	1.4			P		5.2						X									
	524			> 20ppm	P	P			406.0			X	X	X	X							7.522
	525			> 20ppm	P	P			22.3			X	X	X	X							?
	531	3.0			P		POS						X									?
	533	9.5			P	P		13.6					X	X	X			x				?
	537	3.3			P	P	3.6			X			X	X	X							7.8
	538	4.5			P	P	2.8			X			X	X	X							?
	540	1.3			P	P	7.3						X	X	X							19
	542	5.2			P		POS						X									
Bulgaria	527			> 20ppm	P		4.2						X					x				32
	528			> 20ppm	P		5.3						X									3
	529			> 20ppm	P	P			76.0			X	X	X	X							?

Annex 1

Cookies

		ELISA				DISPSTICK	RT PCR			RT PCR-ELISA				RT PCR-DIPSTICK	ELISA- RT PCR-DIPSTICK	ingredients		"may contain"		present in factory		cocoa /choco
	sample no	0.7-10ppm	10-20 ppm	>20 ppm	pos / neg		0.7-10ppm	10-20 ppm	>20 ppm	0.7-10ppm	10-20 ppm	>20 ppm	QUALIT			PEA	NUT	PEA	NUT	PEA	NUT	content %
Germany	10	0.4				P							X					x				?
	29	0.3				P							X									
	54	0.1				P							X									
	57	0.5				P	POS							X			x					
	61	0.3				P							X									
	8	0.0					POS															
Belgium	198	0.0				P							X									11
	203	0.1				P							X						x			6
	204	-0.1				P							X									23
	224	0.2				P							X						x			
	251	-0.2				P							X									48
	254	-0.1				P							X									48
The Netherlands	380	-0.3				P							X					x				39
	381	-0.3				P							X					x				47wh/echoc.
	386	-0.3				P							X					x				15
	398	-0.2				P							X				x	x				30
	374	0.5											X		X				x	x		
Austria	176	0.2				P							X									
	171	-0.2					POS															
Czech Republic	361	-0.2				P							X									28
Poland	434	-0.2				P							X					x				31
Hungary	461	-0.1				P							X						x			
	462	0.2				P							X						x			
	448	-0.2											X	X	X				x			
	440	-0.2											X	X	X							
Slovakia	535	0.2					POS															
Bulgaria																						

Annex I *Cookies*

		ELISA				DISPSTICK	RT PCR			RT PCR-ELISA				RT PCR-DIPSTICK	ELISA- RT PCR-DIPSTICK	ingredients		"may contain"		present in factory		cocoa /choco content %
	sample no	0.7-10ppm	10-20 ppm	>20 ppm	pos / neg		0.7-10ppm	10-20 ppm	>20 ppm	0.7-10ppm	10-20 ppm	>20 ppm	QUALIT			PEA	NUT	PEA	NUT	PEA	NUT	
Germany	39	0.0											X	X	X	x			x			22
Belgium	202			> 20ppm	P	P			4765			X	X	X	X	x						
	236	0.2											X	X	X	x						13
	239	0.2											X	X	X	x						22
	257	0.2											X	X	X	x	x					26
	568												X	X	X	x			x			
The Netherlands																						
Austria																						
Czech Republic	322	0.1											X	X	X	x				x		15
	323	0.2							26			X				x				x		15
	332			>20ppm	P	P			41492			X	X	X	X	x						30
	346			> 20ppm	P	P			339582			X	X	X	X	x			x			7.5
	351			> 20ppm	P	P			186351			X	X	X	X	x						7
Poland																						
Hungary	451	-0.3											X	X	X	x				x		3.6
	452	-0.3											X	X	X	x						?
Slovakia	512			> 20ppm	P	P			60858			X	X	X	X	x						30
	526			> 20ppm	P	P			106087			X	X	X	X	x						7.5
	541			> 20ppm	P	P			225042			X	X	X	X	x						30
Bulgaria																						

Annex I — *Chocolates*

		ELISA				DISPSTICK	RT PCR			RT PCR-ELISA				RT PCR-DIPSTICK	ELISA- RT PCR-DIPSTICK	ingredients		"may contain"		present in factory		cocoa content
	sample no	0,7-10ppm	10-20 ppm	>20 ppm	pos / neg		0,7-10ppm	10-20 ppm	>20 ppm	0.7-10ppm	10-20 ppm	>20 ppm	QUALIT			PEA	NUT	PEA	NUT	PEA	NUT	%
Germany	84	2.6			P	P												x	x			50
	86			>20ppm	P	P			259.0			X	X	X	X			x	x			30
	87			>20ppm	P	P			37.5			X	X	X	X			x	x			50
	89			>20ppm	P	P			29.7			X	X	X	X			x	x			30
	90		20.0		P	P		11.3			X		X	X	X			x	x			30
	98		20.0		P	P	7,1						X	X	X			x	x			30
	99	7.6			P	P												x	x			50
	100		15.0		P	P	9,8						X	X	X			x	x			30
	101	6.4			P	P	POS						X	X	X			x	x			60
	147	3.0			P	P	POS						X	X	X				x			milk
Belgium	122	5.6			P									X					x			30 choc.
	124	0.8			P									X					x			milk/dark 32%
	143	2.7			P									X					x			46
The Netherlands	415	1.5			P		POS						X						x			30
	416	2.7			P		POS						X						x			50
	420	0,7			P									X					x			35
Austria																						
Czech Republic	263	0.7			P		POS						X					x				40
	264			> 20ppm	P	P		11.6					X	X	X			x				40
	265	9.5			P	P												x				40
	266			> 20ppm	P	P	POS						X	X	X			x				40
	267	1.6			P									X				x				40
	268	2.6			P									X				x				40
Poland	548			> 20ppm	P	P		18.2					X	X	X			x	x			30
	549		10.3		P									X				x	x			51
	550			> 20ppm	P	P			118.4			X	X	X	X			x	x			white
	555		11.0		P		8.9						X					x	x			25
	556			> 20ppm	P		POS						X					x	x			77
	558			> 20ppm	P	P			106.0			X	X	X	X							milk
	559	1.9			P	P												x	x			30
	562	1.3			P									X				x				61
	564	1.0			P									X				x	x			64
Hungary	465			>20ppm	P	P			67.7			X	X	X	X			x				28
	466			>20ppm	P		12.6						X					x				52
	467			>20ppm	P	P			601.0			X	X	X	X			x				28
	468		12.8		P									X				x				51
	469			>20ppm	P	P			70.5			X	X	X	X							30
	470	3.6			P		POS						X					x				30
	471	5.8			P		4.8			X			X									30
	472			>20ppm	P		6.8						X					x				52
	474			>20ppm	P	P			61.0			X	X	X	X			x				stracciatella 7%
	475		19.5		P	P		16.5			X		X	X	X			x				25
	476	1.0			P		POS						X					x				25
	477	8.2			P	P		10.4					X	X	X			x				25
	481			>20ppm	P	P			67.7			X	X	X	X			x				30
	483	6.0			P			12.0					X					x				28
Slovakia	485			>20ppm	P	P		13.5					X	X	X			x				62
	485			>20ppm	P	P			125.2			X	X	X	X			x	x			29
	487	0.8			P									X				x	x			48
	488			>20ppm	P	P		15.2					X	X	X			x	x			29
	490			>20ppm	P		5.5						X					x	x			35
	492	8.2			P		POS						X					x	x			28
	493			> 20ppm	P	P			986.3			X	X	X	X			x	x			28
	494		11.9		P	P	9.5						X	X	X			x	x			28
	495			> 20ppm	P	P		14.7					X	X	X			x	x			28
	496			> 20ppm	P	P			917.5			X	X	X	X			x	x			29
	498			> 20ppm	P	P			4658.3			X	X	X	X							
	500			> 20ppm	P	P			84.5			X	X	X	X				x			44
	501			> 20ppm	P	P			21.0			X	X	X	X			x	x			45
	503		15.3		P		6.1						X						x			
	566			> 20ppm	P	P			45.7			X	X	X	X			x	x			
	567			> 20ppm	P	P			1841.2			X	X	X	X			x	x			
Bulgaria	544			> 20ppm	P		1.6						X					x	x			54
	545			> 20ppm	P	P			10682.0			X	X	X	X							
Romania	275	3.1			P									X				x	x			30
	276		17.3		P	P		13.6			X		X	X	X			x	x			30
	277			>20ppm	P	P			246.2			X	X	X	X			x				10
	280			>20ppm	P	P		15.2					X	X	X							31
	281	6.9			P	P	4.0			X			X	X	X							31
	282			>20ppm	P	P	4.9						X	X	X							31
	283	8.0			P	P	POS						X	X	X							31
	284	1.0			P		3.4			X			X					x	x			10
	290	2.5			P									X								stracciatella31%
	301		11.6		P	P		11.8			X		X	X	X			x				25.5
	302			> 20ppm	P	P			57.4			X	X	X	X			x				25.5
	303			> 20ppm	P	P			53.2			X	X	X	X			x				20
	305	6.0			P	P	8.1			X			X	X	X			x				white 20%
	306			> 20ppm	P	P			36.5			X	X	X	X			x				30
	307			> 20ppm	P	P			47.1			X	X	X	X			x				25.5
	308			> 20ppm	P	P			190.2			X	X	X	X			x				10
	309			> 20ppm	P	P			113.6			X	X	X	X			x				10
	310		15.1		P	P	6.7						X	X	X							25
	311			> 20ppm	P	P			106.6			X	X	X	X							25
	312			> 20ppm	P	P			50.1			X	X	X	X							25
	313			> 20ppm	P	P		16.9					X	X	X			x				10
	314			> 20ppm	P	P			23.9			X	X	X	X			x				10
	315			> 20ppm	P	P			126.7			X	X	X	X			x				10
	316			> 20ppm	P	P			36.0			X	X	X	X			x				10
	317			> 20ppm	P	P			45.1			X	X	X	X			x				10

	ELISA				DISPSTICK	RT PCR			RT PCR-ELISA				RT PCR-DIPSTICK	ELISA- RT PCR-DIPSTICK	ingredients		"may contain"		present in factory		cocoa content
	sample no	0,7-10ppm	10-20 ppm	>20 ppm	pos / neg		0,7-10ppm	10-20 ppm	>20 ppm	**0,7-10ppm**	**10-20 ppm**	**>20 ppm**	**QUALIT**			**PEA**	**NUT**	**PEA**	**NUT**	**PEA**	**NUT**	%
Germany																						
Belgium																						
The Netherlands																						
Austria																						
Czech Republic																						
Poland																						
Hungary	484	0,03											X	X	X	x		x				28
Slovakia	489			>20ppm	P	P			15839,2				X	X	X	x			x			28 ?
	497			> 20ppm	P	P			23457,8				X	X	X	x						?
	499			> 20ppm	P	P			29086,9				X	X	X	x			x			29 ?
	565			> 20ppm	P	P			30057				X	X	X	x						29 ?
Bulgaria																						
Romania																						

		ELISA				DISPSTICK	RT PCR			RT PCR-ELISA				RT PCR-DIPSTICK	ELISA- RT PCR-DIPSTICK	ingredients		"may contain"		present in factory		cocoa content
	sample no	0.7-10ppm	10-20 ppm	>20 ppm	pos / neg		0.7-10ppm	10-20 ppm	>20 ppm	**0.7-10ppm**	**10-20 ppm**	**>20 ppm**	**QUALIT**			**PEA**	**NUT**	**PEA**	**NUT**	**PEA**	**NUT**	%
Germany	62	0.17											X	X	X				x			60
Belgium	127	-0.16											X	X	X			x	x			white choc.
The Netherlands	414	-0.01											X	X	X					x	x	29 white choc.
	408	0.5											X	X	X						x	31
Austria	167	-0.14											X	X	X							34
Czech Republic	262	-0.04											X	X	X							30
Poland	563	-0.19				P							X					x	x			28
	554	0.6											X	X	X			x	x			dark choc.?
Hungary	473	0.45											X	X	X			x				30
	479	0.6					POS											x				white choc.
Slovakia																						
Bulgaria	543	-0.03											X	X	X			x				48.8
Romania	304	0.23											X	X	X			x				25.5

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Studies

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1998-2003: Degree in Food Sciences and Technologies (5 years); Facolta' di Agraria - Universita' degli Studi di Parma (Italy). Title of the thesis: "Hidden Allergens in Foodstuff: PCR and PNA methodologies for Detection of Hazelnut and Peanut"; Overall marks: 110/110 cum laude.

2003: Qualification for practising the profession of food technologist (Esame di Stato).

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Publications:

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Oral presentation

"Detection of peanut allergen residues in processed food products: the development of three real-time PCR assays compared with ELISA". Euronalysis XIV, 10-14 Settembre 2007; Antwerp (Belgium).

"Development of new real-time PCR assays for food allergen detection". Life Sciences Symposium at IRMM, 15 Gennaio 2008; IRMM, Geel Belgium.