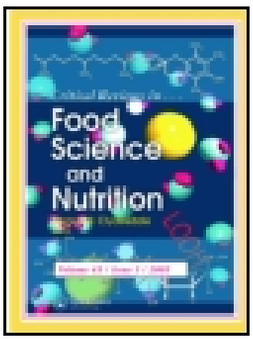

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REVIEW

Application of real-time PCR for tree nut allergen detection in processed foods

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ABSTRACT

Currently, food allergies are an important health concern worldwide. The presence of undeclared allergenic ingredients or the presence of traces of allergens due to accidental contamination during food processing poses a great health risk to sensitized individuals. Therefore, reliable analytical methods are required to detect and identify allergenic ingredients in food products. Real-time PCR allowed a specific and accurate amplification of allergen sequences. Some processing methods could induce the fragmentation and/or degradation of genomic DNA and some studies have been performed to analyze the effect of processing on the detection of different targets, as thermal treatment, with and without applying pressure. In this review, we give an updated overview of the applications of real-time PCR for the detection of allergens of tree nut in processed food products. The different variables that contribute to the performance of PCR methodology for allergen detection are also review and discussed.

KEYWORDS

Real-time PCR; tree nut; allergen detection; processed foods; thermal processing; pressure processing;

1. Introduction

Food allergy is considered a relevant health problem all over the world, and its prevalence is difficult to establish because it varies by country and food, being estimated to affect close to 5% of adults and 8% of children (Sicherer and Sampson 2014). The most common foods associated with allergenic reactions are fish, peanuts, soybeans, milk, eggs, crustacean, wheat and tree nuts. Tree nuts are valuable foods rich in proteins, minerals, vitamins, antioxidants and a considerable high content of unsaturated fatty acids (Ros 2010), and their global production and consumption is increasing in last years. Tree nut allergy has also experienced an increase, although prevalence varies among individual nut and regions being difficult to establish it. In Europe, eventhough the most common allergies to tree nuts correspond to hazelnut and walnut, cashew allergy is getting importance over the last years according to several reports, with a variable prevalence among countries (Mendes et al. 2016). Other studies indicate that allergy to walnut and cashew is the most prevalent among tree nuts in USA, achieving 20-30% and 15-30% respectively (McWilliam et al. 2015; Weinberger and Sicherer 2018).

In order to protect the security of the allergic patients, European regulation obliges to indicate the presence of tree nuts in food labels (Official Journal of the European Union (2011)). Presence of potentially allergenic ingredients in foods can be a consequence of fraudulent substitution or adventitious contamination in the food facility. Most of food industries have shared production lines for nut processing, therefore the routes associated with potential cross-contamination (namely people's handling, raw material handling, transport, processing aids, packing) with other nut or foods cannot be neglected.

There is no treatment for food allergies, and sensitized individuals have to totally avoid the consumption of offending ingredients. Therefore, to develop a reliable and specific tool to detect traces of specific food allergens is indeed essential to improve the quality of life of sensitized individuals. Among the immunoassays,enzyme-linked immunosorbent assay (ELISA) is the most used and common method to detect small amounts of proteins from specific foods and it is possible to find several ELISA kits, as well as other commercial immunoassays (i.e., lateral flow) in the market. One of the inconvenients of the immunoassays is that protein solubility can be affected by food processing, and the subsequent detection with protein-based techniques might be altered (Mattison et al. 2016). DNA-based methodologies, such as real-time polymerase chain reaction (PCR), have been proposed as an specific, sensitive and reliable alternative to ELISA, since DNA molecules maintain their integrity better than proteins (Sun et al. 2012). Several studies have been carried out to develop real-time PCR methods for detecting allergens in foods (Prado et al. 2016; Costa et al. 2017a), using different approaches and chemistries, and obtaining promising results.

The present review gives an updated overview of the applications of real-time PCR for the detection of tree nuts in processed food products. The different variables that

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Table 1. Effects of processing on IgE-reactivity/antigenicity of specific tree nut allergens.

Food	Allergen	Processing conditions	IgE reactivity/ antigenicity analysis*	Reference
Almond	PE	Boiling, 100 °C, 5 and 10 min	=	Su et al. 2004
	PE	Frying, 191 °C, 1 min	= (∼↓)	Su et al. 2004
	PE	Roasting 160 °C, 30 min; 177 °C, 12 min	=	de Leon et al. 2003
	PE	Roasting 180 °C, 15 min	=	Su et al. 2004
	PE	Autoclaving 121 °C, 30 min	=	Venkatachalam et al2002, Su et al2004
Brazil nut	PE	Frying, 191 °C, 1 min	=	Sharma et al. 2009
	PE	Roasting up to 160 °C, 30 min; up to 177C, 12 min	=	Sharma et al. 2009
	PE	Roasting 180 °C, 15 min	=	de Leon et al. 2003
	PE PE PE	Autoclaving 121 °C, 30 min	=	Su et al. 2004
	PE PE PE	Autoclaving 121 °C, 30 min	= (∼↓)	Sharma et al. 2009
Cashew	Ana o 1, 2, 3	Boiling, 100 °C, 10 min	∼↓	Venkatachalam et al. 2002
	PE	Boiling, 100 °C, 30 and 60 min	= (∼↓)	Cuadrado et al2018, Sanchiz etal2018a
	PE	Frying, 191 °C, 1 min	= (∼↓)	Su et al. 2004
	Ana o 1, 2, 3	Roasting 200 °C, 15 min	∼↑	Venkatachalam et al. 2002
	PE	Roasting 180 °C, 15 min	=	de Leon et al. 2003
	Ana o 1, 2, 3	Autoclaving 121 °C, 30 min	↓	Venkatachalam et al. 2008
	PE	Autoclaving 121 °C, 30 min	↓	Su et al. 2004
	PE	Autoclaving 138 °C, 15 and 30 min	↓↓	Cuadrado et al2018, Sanchiz et al. 2018a
Hazelnut	PE	Roasting 140 °C, 40 min	↓	Hansen et al. 2009
	PE	Roasting 144 °C, time not indicated	↓	Worm et al. 2009
Pecan	PE	Roasting 137 °C, 30 min; 148 °C, 30 min; 160 °C, 30 min; 168 °C, 12 min; 176 °C, 12 min	=	Venkatachalam et al. 2006 (Venkatachalam et al. 2006)
	PE	Autoclaving 121 °C, 30 min	∼↓↓	Venkatachalam et al. 2006
Pine nut	Pin p 1	Boiling, 100 °C, 2h	=	Cabanillas et al. 2016
Pistachio	PE	Boiling, 100 °C, 30 and 60 min	= (∼↓)	Cuadrado et al. 2018, Sanchiz et al. 2018a
	PE	Autoclaving 138 °C, 15 and 30 min	↓↓	Cuadrado et al2018, Sanchiz et al. 2018a
Walnut	PE	Frying, 191 °C, 1 min	= (∼↓)	Su et al. 2004
	PE	Roasting 160 °C, 30 min; 177 °C, 12 min	=	Su et al. 2004
	PE and Jug r 4	Autoclaving 138 °C, 15 and 30 min	↓↓	Cabanillas et al. 2014
	PE and Jug r 4	HHP 300-600 MPa, 15 min	=	Cabanillas et al. 2014

*IgE reactivity/antigenicity measured by different techniques and conditions. Characteres: =, ↓,↑, ∼, are a symbolic representation of the global effect of the specific treatment on the IgE-reactivity/antigenicity of a given food (=, similar;↑ increase; ↓,decrease;,∼↑ slight increase; ∼↓, slight decrease). PE: protein extract.

contribute to the performance of PCR methodology, as well as new advances in DNA-based methodologies for allergen detection, are also review and discussed.

2. Food processing and allergenicity

Consumers buy ready-to-eat processed foods for several reasons, being convenience perhaps the most important one. A specific food can be subjected to a wide variety of treatments, whether at home, institutional, or industrial setting; for different reasons: (1) improvement of food qualities such as flavor, texture, taste, color; (2) improve preservation and safety; (3) enhance suitability for specific product applications; (4) convenience, pleasure and variety; (5) obtain or generate useful by-products; and (6) increase marketability and/or revenue. Tree nuts are normally consumed as snacks or as ingredient in foodstuff. As other foods, are subjected to daily processing to improve their quality, preservation, safety and suitability for specific product applications. The degree of processing affects the digestibility, solubility and other related parameters. Food processing can also lead to a wide variety of biochemical reactions among the different components of foods. Foods are complex mixtures of proteins, sugars, fats, water, etc, and such biochemical reactions have the potential to modify the allergenicity of foods. One major factor responsible for the formation of some neoantigens is the Maillard reaction, i.e., the interaction of protein component with sugar residues upon heating, generating sugar conjugated protein derivatives, which enhance the allergenicity of protein (Maleki 2004).

During processing, proteins can form oligomers, become denatured, aggregated, fragmented and re-assembled and these changes most often reduce solubility (Maleki 2004). Processing can alter the overall IgE binding profiles of a particular extract becoming more or less antigenic or resulting in new allergens (neoallergens) (Schmitt et al. 2010). The degree of such modifications depends on different factors such as the type of processing applied (i.e., thermal or non-thermal), the processing conditions, time, environment, etc. (Cabanillas and Novak 2017).

Table 2. Analysis of the technological processing effect on technique's ability to identify tree nut allergen DNA by polymerase chain reaction.

Food	DNA target	Amplicon size (pb)	Processing conditions	Assay performance	Processing analysis	Reference
Almond	ITS 1	76	Roasting 160 °C 13 min	=	Binary mixture in wheat	López-Calleja et al. 2014a
	Pru du 3	100–102	HHP 300, 400, 500, 600, 15 min	=	DNA normalization	Prieto et al. 2014
	Pru du 3	100–102	Autoclaving 121 °C and 138 °C, 15 and 30 min	–	DNA normalization	Prieto et al. 2014
Brazil nut	Ber e 1	131	Roasting 160 °C 13 min	=	Binary mixture in wheat	De la Cruz et al. 2013
Cashew	Ana o 1	65	Boiling 30, 60 min	=	Binary mixture in wheat	Sanchiz et al. 2018b
	Ana o 1	65	Autoclaving 121 °C 15 and 30 min; 138 °C 15 min	↓ sensibility	Binary mixture in wheat	Sanchiz et al. 2018b
	Ana o 1	65	Autoclaving 138 °C 30 min	–	Binary mixture in wheat	Sanchiz et al. 2018b
	ITS 1	69	Roasting 160 °C 13 min	=	Binary mixture in wheat	López-Calleja et al. 2015a
Hazelnut	ITS 1	70	Roasting 160 °C 13 min	=	Binary mixture in wheat	López-Calleja et al. 2013
	Cor a 9, 11, 13	101	HHP 400, 500, 600, 15 min, 20 °C	=	DNA normalization	Iniesto et al. 2013
	Cor a 9, 11, 13	101	Roasting 180 °C 20-30 min	↓ sensibility	DNA normalization	Iniesto et al. 2013
	Cor a 9, 11, 13	101	Autoclaving 121 °C and 138 °C 15 and 30 min	↓ sensibility	DNA normalization	Iniesto et al. 2013
	Cor a 8	218	Baking 180 °C 16 min	↓ sensibility	Model pastry - cookies	Platteau et al. 2011
Macadamia	ITS 1	72	Roasting 160 °C 13 min	=	Raw/heated nut in wheat. CFP	López-Calleja et al. 2015a
Pecan	ITS 1	69	Roasting 160 °C 13 min	=	Raw/heated nut in wheat. CFP	López-Calleja et al. 2015b
Pistachio	Pis v 1	81	Boiling 30, 60 min	=	Binary mixture in wheat	Sanchiz et al. 2017
	Pis v 1	81	Autoclaving 121 °C 15 min	↓ sensibility	Binary mixture in wheat	Sanchiz et al. 2017
	Pis v 1	81	Autoclaving 121 °C 30 min	↓ Eff, R^2	Binary mixture in wheat	Sanchiz et al. 2017
	Pis v 1	81	Autoclaving 138 °C 15 and 30 min	–	Binary mixture in wheat	Sanchiz et al. 2017
	ITS 1	71	Roasting 160 °C 13 min	=	Binary mixture in wheat	López-Calleja et al. 2014b
Walnut	Jug r 1, 3, 4	91–99	HHP 300, 400, 500, 600, 15 min, 15 °C	=	DNA decimal dilution	Linacero et al. 2016
	Jug r 1, 3, 4	91–99	Autoclaving 121 °C 15 min	↓ sensibility	DNA decimal dilution	Linacero et al. 2016
	Jug r 1, 3, 4	91–99	Autoclaving 121 °C 30 min	↓ Eff, R^2	DNA decimal dilution	Linacero et al. 2016
	Jug r 1, 3, 4	91–99	Autoclaving 138 °C 15 and 30 min	–	DNA decimal dilution	Linacero et al. 2016
	ITS 1	70	Roasting 160 °C 13 min	=	Binary mixture in wheat	López-Calleja et al. 2015b
	Jug r 3	99	Baking 200 °C 20 min	=	Model pastry - cake	Costa et al. 2013b

N.D. not determined; = similar compared to raw material; ↓ refers to: lower sensibility or Eff out from 90 to 110% range and R^2 below 0.98; -: detection/quantification not viable.

In addition to the effect of food processing technologies, the different nature of allergens (profilins, globulins, albumins, pathogenesis-related proteins, lipid transfer proteins) is determinant to increase or reduce the allergenicity of foods (Sathe et al. 2005; Mills et al. 2009). Until the date, the effects of food processing technologies on allergenic properties of food products have been studied for several tree nuts (Masthoff et al. 2013). Some studies have investigated the influence of boiling and frying on the IgE-binding resistance of purified allergens and protein extract from certain tree nuts. Antigenicity of pine nut, walnut, almond, cashew and pistachio proteins was almost unaltered after boiling (Cabanillas et al. 2016; Su et al. 2004; Cuadrado et al. 2018; Sanchiz et al. 2018a) or frying (Su et al. 2004). The effect of roasting in tree nuts such as hazelnut has been found to depend on the specific pattern of allergic sensitization of the patients (Hansen et al. 2009; Worm et al. 2009). In other tree nuts such as walnut, proteins such as 2S albumin or LTP had little changes in their abundance after roasting, however the mature 7S globulin and 11S globulin was significantly higher detected after roasting at 180 °C for 20 minutes (Downs et al. 2016). However, tree nuts such as cashew, almond, pecan or Brazil nut showed stability of antigenicity after roasting (Venkatachalam et al. 2002, 2008; Sharma et al. 2009; Su et al. 2004). The combination of pressure and heat at specific conditions during autoclaving at harsh conditions (2.8 atm (37 psi), 138 °C for 15 or 30 minutes) were found to lead to fragmentation of walnut,

cashew and pistachio proteins that went along with a reduced IgE-binding and IgE crosslinking capacity. The results were confirmed by different in vitro and in vivo techniques (Cabanillas et al. 2014; Cuadrado et al. 2018; Sanchiz et al. 2018a). Interestingly, other food treatments that involve high pressure and low temperature did not have the same effect as autoclave conditions (Cabanillas et al. 2014; Husband et al. 2011). Therefore, the combination of heat and pressure during autoclaving seems to be key for protein degradation and potential decrease in IgE-reactivity. Table 1 summarizes the effects of processing on IgE-reactivity/antigenicity of specific tree nut allergens.

The effect of technological processing on protein structure usually represent one of the major problems associated with the detection of allergenic proteins in processed foods by immunological methods (Platteau et al 2011a). At the same time, other molecules from food products are also affected and DNA is not an exception; consequently, the effects that food processing might exert on DNA molecules is also an important issue to analyze. There are several reports where a negative effect on DNA detection after severe heat treatment has been described (Scaravelli et al. 2009; Costa et al. 2013b; Iniesto et al. 2013). Some processing methods could induce the fragmentation and/or degradation of genomic DNA (Gryson 2010; Ballari and Martin 2013; Prieto et al. 2014) and some studies have been performed to analyze the effect of processing on the detection of different targets, as thermal treatment, with and without applying pressure (boiling, high hydrostatic pressure HHP, autoclave, frying, roasting), in peanut, hazelnut, walnut, almond and pistachio (Table 2). Thermal treatment combined with pressure (autoclaving) reduced yield and amplificability (integrity and quality) of tree nut DNA. High hydrostatic pressure (HHP) did not produce any effect on the tree nut DNA amplificability (Scaravelli et al. 2009; Iniesto et al. 2013; Prieto et al. 2014; Linacero et al. 2016; Sanchiz et al. 2017, 2018b). When comparing with the respective set of raw mixtures, it is clear that processing did not affect the sensitivity and quantification. However, for some concentration levels there were significant differences between Ct values, mainly in autoclaved mixtures. This finding suggests the necessity of using calibration curves prepared with reference mixtures submitted to similar thermal processing applied to samples (particularly in the case of autoclaved ones) (Costa et al. 2017b).

3. DNA methodologies for allergen detection

Methodologies based on genetics and molecular biology have become interesting approaches for tracking down the presence of trace amounts of allergens at any stage along the food supply chain (Di Bernardo et al. 2005, 2007). DNA-based technology is a relatively new approach for the detection of food allergens, a field traditionally dominated by immunoassays. DNA assays have been used for food authentication, including speciation, and for the detection of products of agricultural biotechnology as genetically modified organisms (GMO) to ensure label accuracy and compliance with regulations. DNA methods are indirect methods to detect food allergens as they do not detect the allergenic protein itself, but the DNA sequences from the allergenic food ingredient. Detection of DNA from foodstuff implies some limitations. The low amount of the target DNA in most food matrices requires a sensitive and specific methodology (Broeders et al. 2012). Usually, DNA methods are based on the amplification of specific DNA fragments either by means of end point PCR, which offers a qualitative result (i.e., it detects the presence of a specific DNA sequence), or by real-time PCR, which gives a quantitative account. In this sense, the PCR, together with real-time PCR, can be a valuable tool to indicate the presence or absence of a particular allergen in food (even below a concentration of 10 ppm). These DNA based analytical methods are fast, sensitive, and provide specific detection of the targeted DNA.

Advantages of DNA-based methods over protein-based methodologies rely in that the target DNA is efficiently extracted from raw and cooked products and is slightly affected by the heating process, because DNA typically remains fragmentated but detectable after being exposed to the cooking temperatures of most foods. Although some components from food matrix may interfere with the PCR or real-time PCR assay, and therefore reduce the amplification efficiency, PCR inhibitors can be removed during DNA extraction and purification steps with the help of suitable DNA clean-up procedures (López-Calleja et al. 2007).

Another important step in the development of methods for the detection of allergenic ingredients in food products is the robustness of the method of analysis to provide reliable analytical results. In this sense, method validation is an essential part of providing reliable results that can be comparable among different laboratories (van Hengel 2007; Monaci and Visconti 2010). Different attempts are being made to establish guidelines for single and interlaboratory validation (Kerbach et al. 2009; Abbott et al. 2010); that can be followed by research groups working on method development. Quantification of allergens, either by immunological or by DNA-based methods, is a challenging problem, mainly due to the unavailability of certified reference materials (CRMs). CRMs are materials or substances whose property values are sufficiently homogenous and well established to be used for method development and validation, or for proof of method performance, and possess certificate and traceable assigned values (to an international system, method or artifact). The use of these CRMs has in general contributed to facilitate compliance with food labeling laws and to improve the accuracy of analytical methods. However, there is a current lack of reference materials for the development of allergen detection methods, which has prompted the scientific community to proactively work on this regard. Up to date, only a CRM has been developed for the detection of food allergens, and only for peanuts. Several research networks are currently involved in the production of food allergen reference materials, like egg powder and skimmed milk powder provided by NIST (National Institute of Standards and Technology) (Kerbach et al. 2009). Even though reference materials are commercially available for

Table 3. Summary of real-time PCR based methods for tree nuts specific detection.

Food	Technique/Chemistry	DNA target	LOD	Dynamic range*	Food mixture/matrix	Reference
Almond	TaqMan probe	ITS 1	0.1 mg/kg	10^6-0.1 mg/kg	Raw/heated nut in wheat. CFP	López-Calleja et al. 2014a
	Sybr Green	Pru du 1, 3, 4, 6	1 mg/kg	10^6-1 mg/kg	Peanut flour, CFP	Prieto et al. 2014
	Nested-Real Time PCR	Pru du 6	50 mg/kg	10^6-50 mg/kg	Walnut	Costa et al. 2013b
	TaqMan probe	nsLTP	5 mg/kg	qualitative	Chocolates/ cookies. CFP.	Röder et al. 2011
	TaqMan probe (multiplex)	Pru av 1	100 mg/kg	qualitative	CFP	Köppel et al. 2010
	Sybr Green	Pru du 1, rbcL	1 copy	qualitative	CFP	Pafundo et al2009
Brazil nut	TaqMan probe	Ber e 1	2.5 mg/kg	10^5-100 mg/kg	Raw/heated nut in wheat. CFP.	De la Cruz et al. 2013
	TaqMan probe	2S albumin	5 mg/kg	qualitative	Cookies, chocolate, dough. CFP	Röder et al. 2010
	TaqMan probe	11S globulin	1000 mg/kg	qualitative	Walnut paste. CFP	Brezna et al. 2010
Cashew	LNA and TaqMan probes	Ana o 1	10 mg/kg	10^6-5 mg/kg	Raw/Boiled/AU nut in wheat. CFP.	Sanchiz et al. 2018b
	TaqMan probe	ITS 1	0.1 mg/kg	10^6-0.1 mg/kg	Raw/heated nut in wheat. CFP	López-Calleja et al. 2015a
	TaqMan probe (multiplex)	Ana o 3	32; 5 (rice) mg/kg	qualitative	Boiled sausages or rice cookies. CFP	Köppel et al. 2012
	SybrGreenER (multiplex)	Ana o 1	0.5 pg	qualitative	Different nuts. CFP.	Pafundo et al. 2010
	TaqMan probe	Ana o 3	2 mg/kg	qualitative	Basil pesto	Ehlert et al. 2009
	TaqMan probe	Ana o 3	100 mg/kg	qualitative	Pistachio nougat, CFP	Piknová and Kuchta2007
	TaqMan probe	Ana o 3	100 mg/kg	qualitative	Chocolate cookies	Brzezinski2006
Hazelnut	TaqMan probe	Hsp 1	50 mg/kg	5.10^6-50 mg/kg	Chocolate	Costa et al. 2014
	TaqMan probe	ITS 1	0.1 mg/kg	10^6-0.1 mg/kg	Raw/heated nut in wheat. CFP	López-Calleja et al. 2013
	Sybr Green	Cor a 9, 11, 13	1 mg/kg	qualitative	Peanut flour, CFP	Iniesto et al. 2013
	TaqMan probe (multiplex)	Cor a 1	100, 5 (rice) mg/kg	qualitative	Sausages/rice cookies, CFP	Köppel et al. 2012
	Nested-Real Time PCR	Hsp 1	50 mg/kg	10^6-50 mg/kg	Wheat flour, CFP	Costa et al. 2012b
	Sybr Green	Cor a 1, 8, 14	10 mg/kg	qualitative	Wheat flour, CFP	D'Andrea et al. 2011
	TaqMan probe (multiplex)	Cor a 1	100 mg/kg	qualitative	CFP	Köppel et al. 2010
	TaqMan probe (multiplex)	Cor a 1	50 mg/kg	qualitative	Cookies, CFP	Schöninghumer et al. 2009
Macadamia	TaqMan probe	ITS 1	0.1 mg/kg	10^6-0.1 mg/kg	Raw/heated nut in wheat. CFP	López-Calleja et al. 2015a
	TaqMan probe	Vicilin	200 mg/kg	qualitative	Model pastry. CFP	Brezna et al. 2009
Pecan	TaqMan probe	ITS 1	0.1 mg/kg	10^6-0.1 mg/kg	Raw/heated nut in wheat. CFP	López-Calleja et al. 2015b
	TaqMan probe	Vicilin	100 mg/kg	qualitative	Model pastry	Brezna and Kuchta 2008
Pine nut	TaqMan probe	Chloroplast tRNA-Leu	0.1 mg/kg	qualitative	Spiked in pesto. CFP	Garino et al. 2016
Pistachio	Sybr Green	Pis v 1	100 mg/kg	10^6-100 mg/kg	Raw/boiled/AU nut in wheat. CFP	Sanchiz et al. 2017
	LNA probe	Pis v 1	10 mg/kg	10^6-100 mg/kg		
	TaqMan probe	ITS 1	0.1 mg/kg	10^6-0.1 mg/kg	Raw/heated nut in wheat. CFP	López-Calleja et al. 2014b
	TaqMan probe	ITS	4 mg/kg	qualitative	Cookies. CFP	Brezna et al. 2008
Walnut	SybrGreen	Jug r 1, 3, 4	100 (Jug r 3) mg/kg	10^6-100 mg/kg	Wheat flour. CFP	Linacero et al. 2016
	TaqMan probe	ITS 1	0.1 mg/kg	10^6-0.1 mg/kg	Raw/heated nut in wheat. CFP.	López-Calleja et al. 2015b
	Nested Real-time PCR	Jug r 3	50 mg/kg	5.10^6-50 mg/kg	Batter and sponge cakes.	Costa et al. 2013a
	TaqMan probe (multiplex)	Jug r 2	100, 20 (rice)	qualitative	Boiled sausages/rice cookies. CFP	Köppel et al. 2012
	SybrGreenER (multiplex)	Jug r 1	0.5 pg	qualitative	Different nuts. CFP	Pafundo et al. 2010
	TaqMan probe	Jug r 2	100 mg/kg	qualitative	Model pastry. CFP	Brezna et al. 2006

CFP = commercial food products. AU = autoclave. *Lineal range on the calibration curve from binary food mixtures.

the most important food allergens, the results obtained may not be comparable as these are not certified. In this sense, the development of reference materials and, ideally, CRMs particularly in different matrices, is highly desirable for the validation of methods of analysis of allergenic ingredients in food (Poms et al. 2010; Johnson et al. 2011).

4. Real-time PCR

The PCR, especially real-time PCR, is a very useful tool for allergen detection and consequently, for the allergenic risk management. It is an indirect method to detect allergens (proteins), or traces of any allergenic sources, through the

Table 4. Common DNA extraction methods for food matrices.

Extraction method	Technology	Pros and cons	References
CTAB based	Selective precipitation	Low cost, high DNA purity. Time consuming, low DNA yield	Gryson et al. 2004 Mafra et al. 2008 Pafundo et al. 2010 Röder et al. 2011
DNeasy Plant mini kit	Column-based system, Silica membrane	Easy of use, high quality DNA Optimum for vegetable products	Hird et al. 2003 D'Andrea et al. 2011
Genespin	Column-based system, Silica membrane	Easy of use, High DNA yield and purity High cost	Mafra et al. 2008
NucleoSpin® Food kit	Column-based system. Silica membrane	High DNA yield, good PCR results for complex food matrices. Low DNA purity	Hird et al. 2003 Mafra et al. 2008 Soares et al. 2010 Costa et al. 2015
PowerPlant Pro Kit	Column-based system, Silica membrane	Easy of use, high quality DNA, fast, elimination of polyphenols/polysaccharides High cost	Iniesto et al. 2013 Linacero et al. 2016
Wizard® Magnetic DNA Purification System for Food	Mobile Solid phase, magnetic particles	Easy of use, high quality DNA High cost, Negative PCR results for complex matrices	Gryson et al. 2004 Di Pinto et al. 2007 Costa et al. 2015

detection of the specific DNA coding sequence of an allergenic protein or of any protein from an allergenic source. The conventional or end-point PCR detect the presence or not of the allergenic source (qualitative) whereas the real-time PCR is more informative, specific and sensitive since provides the quantitative results.

High number of PCR assays have been reported for the detection of tree nut traces in processed products. The application of real-time PCR in the detection of different nuts is shown in Table 3. It shows the technique, chemistry and the target sequence used in each report. The limit of detection (LOD) and the application on food matrices is indicated as well as if a quantitative analysis has been reported. Most of the works refer to the use of real-time PCR although other techniques are shown such as nested-real-time PCR (Costa et al. 2012a, 2013b). On the other hand when the technique has been real-time PCR indicates the type of chemistry, Sybr Green (Pafundo et al. 2009, 2010; D'Andrea et al. 2011; Iniesto et al. 2013; Prieto et al. 2014), hydrolysis probes as LNA (Sanchiz et al. 2017) or TaqMan (Brezna et al. 2009; Piknová and Kuchta 2007; Ehlert et al. 2009; Schöringhumer et al. 2009, Köppel et al. 2010). The target are multicopy sequences such as ITS (Brezna et al. 2010; López–Calleja et al. 2013, 2014a, 2015a), chloroplast sequences (Garino et al. 2016) or single/low copy sequences as the genes that encode different allergens (Costa et al. 2013a, b; Köppel et al. 2012; D'Andrea et al. 2011; Brezna et al. 2009; Sanchiz et al. 2017)

The success of a PCR-based method to detect DNA from an allergenic source in a food product depends of three aspects that have to be optimized: DNA quality, primer and probe design specificity and sensitivity.

4.1. DNA extraction and purification

Food products represent a complex matrix containing DNA in a mixture with various compounds (fat, polysaccharides, polyphenols, proteins …). DNA in food is often fragmented or degraded, and the accompanying compounds may cause PCR inhibition if they manage to pass through the DNA isolation procedure. DNA isolation deserves adequate attention because it can strongly influence the results of the downstream PCR. Besides the high efficiency, DNA isolation methods should be as simple and fast as possible.

Two methods are the most widely used for DNA isolation from foods: chaotropic solid phase extraction (SPE) and selective precipitation with CTAB (cetyltrimethylammoinium bromide) and liquid-liquid extraction. The chaotropic SPE method, based in the binding of DNA to a chaotropic salt which it is bound to a silica matrix, is the faster and less laborious alternative, there are several commercial kits based on this methodology (Table 4). Previous works published on food analysis highlighted that it is of key importance to determine the effective method that warranted the best performance of allergen detection based on real-time PCR. The CTAB agent promoted the selective precipitation of DNA and RNA while others compouds, such as polysaccharides, phenolic and other PCR-inhibitors are soluble. This method is more laborious, but more effective than SPE to remove possible PCR-inhibitors, although the DNA yield may be lower (Zimmermann et al. 1998). In some experiments DNA was extracted using the CTAB based method combined with a solid phase extraction isolation kit (Iniesto et al. 2013; Prieto et al. 2014).

There are several works comparing the efficiency of various DNA extraction methods from food matrices and evaluating the effective application in real-time PCR assay (Hird et al. 2003; Di Pinto et al. 2007; Mafra et al. 2008; Kenk et al. 2012; Iniesto et al. 2013; Costa et al. 2015) showing different results depending on the complexity and processing of the food matrix. Costa et al. (2015) compared seven DNA extraction methods for chocolate matrices and assesed the extracts for their suitability for amplification by qualitative PCR and real-time PCR. CTAB-PVP method, Wizard® Magnetic DNA Purification System for Food and Wizard® DNA purification resin based methods yielded the lowest DNA content but the highest level of purity. On the other hand, the extract from NucleoSpin® Food kit presented more DNA but not good purity. However, the best qualitative and real-time PCR results were obtained with Nucleo Spin followed by CTAB-PVP method for the detection of

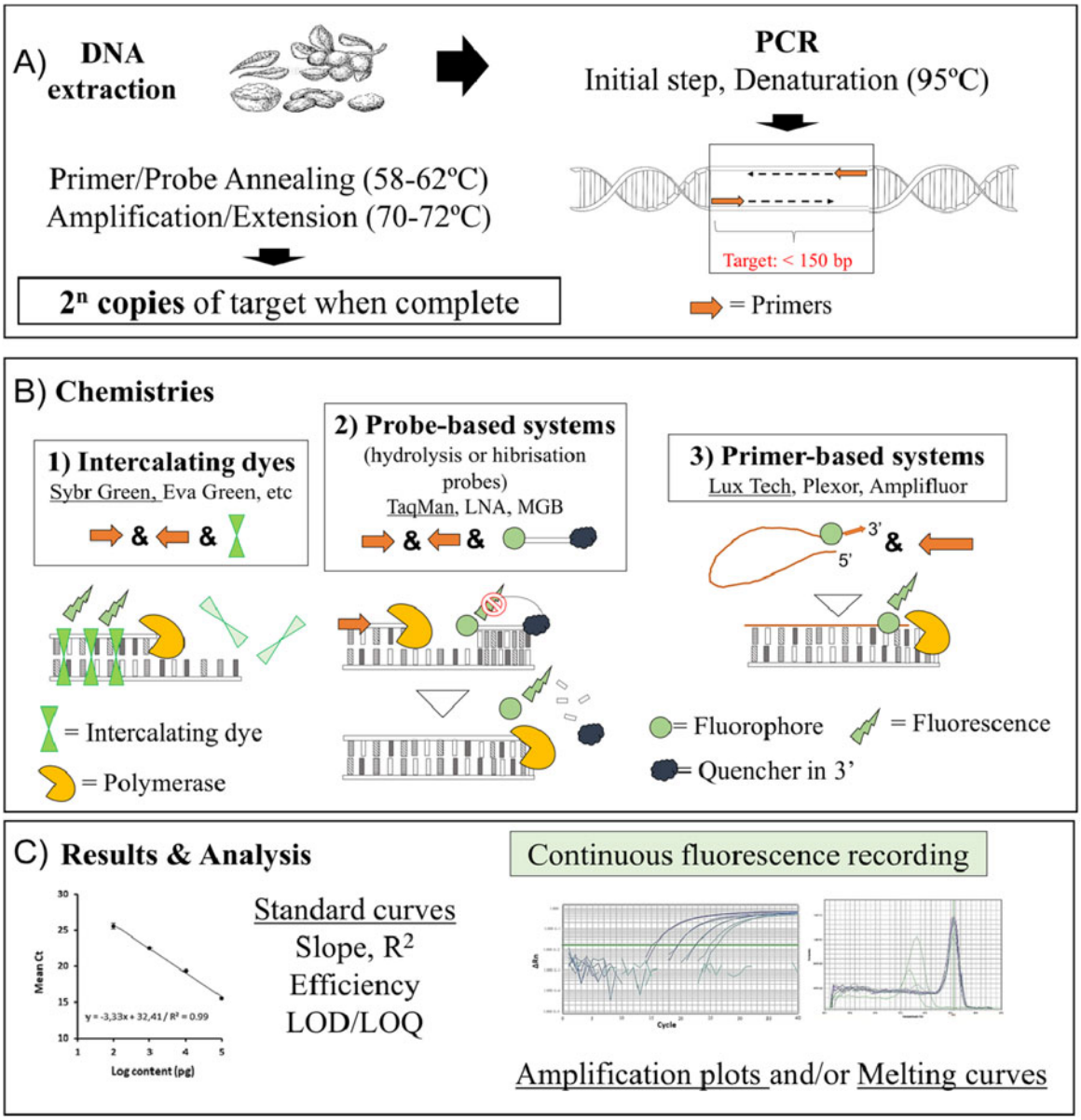

Figure 1. Schematic representation of a real time PCR experiment and typical chemistries. A) Steps of real time PCR B) Available chemistries for real time PCR experiments. Represented technology is underlined, e.g., TaqMan based-chemistry C) Representation of final data from a real time PCR experiment.

almond and hazelnut in chocolate matrices. Similar results were showed by Di Pinto et al. (2007) in the evaluation of DNA isolation from different food matrices with Wizard® Magnetic DNA Purification System and DNeasy® Tissue Kit. They obtained positive results by PCR amplification for all samples except chocolate cream and maize oil. Nevertheless, other experiments obtained high quality DNA from complex matrices, such as chocolate bars and cookies, with CTAB based methods (Gryson et al. 2004; Pafundo et al. 2010; Röder et al. 2011). CTAB method is laborious and time consuming while commercial kits are faster, less technically demanding and optimized to give highest yield of DNA.

4.2. Specificity and sensitivity

End-point PCR is the simplest and, most likely, the least expensive approach for DNA amplification. In this case, the amplified product can be qualitatively visualized by staining after agarose gel electrophoresis, providing information on the size of the amplified product as is the case of the end point PCR. The real-time PCR is a type of PCR where data are collected in "real time" as the reaction proceeds. Real-time PCR is a very sensitive technique, detecting down to a few molecules of target DNA. In contrast to classical PCR, the fluorescence measurements allow detection and quantifying the amplified products of the polimerase reaction. The quantification of the amplification is based on the measurement of a fluorescent signal that increases proportionally as the PCR products are generated.

Real-time PCR assay can be performed using several chemistries commercially available (Figure 1B): intercalating dyes, probe-based or primer-based chemistries. Sybr-Green is one of the most used intercalant dyes. It is a fluorochrome dye that binds double-stranded DNA molecules by intercalating between the DNA bases. These will bind non-specifically to DNA and allow detection of double-stranded

DNA because the fluorescence can be measured at the end of each amplification cycle, in order to determine how much DNA has been amplified. Many works describing a real-time PCR method to detect tree nut allergens have been reported with Sybr-Green as intercalating dye (Pafundo et al. 2010; Prieto et al. 2014; Linacero et al. 2016). The sensitivity of Sybr-Green is adequate in some nuts as almond, hazelnut or walnut. The LOD described, using as target allergen-coding sequences, is similar to that obtained by using hydrolysis probes. In other nuts, as pistachio, the use of probes gave better results in terms of sensitivity (Table 3). However the lack of specificity of this detection chemistry can be considered as a drawback, and probe-based PCR is a suitable alternative for detecting specific sequences due to the incorporation of a third DNA sequence, the probe, which anneal in the sequence flanked by the two primers (Sanchiz et al. 2017). The probe-based PCR relies on the use of specific probes of the sequence to be detected. Probes can be based on hydrolysis or hybridation. First ones are also called 5'-nuclease or hydrolysis probes, commonly located between the PCR primers as fluorescence oligonucleotides they have a fluorophore at its 5 'end and an inhibitor or quencher at 3' (Figure 1B). When real-time PCR is performed, probe hybridizes with the complementary region of the amplicon, but due to the proximity of the fluorophore to the inhibitor, no fluorescence is emitted. However when the polymerase reaches the position of the probe hydrolyzes it through its 5′-3′exonuclease activity, which causes separation of the fluorochrome inhibitor and, therefore, the emission of fluorescence that is related to the amount of amplicon produced (Valasek and Repa 2005). Classical TaqMan or some variants as Locked Nucleic Acid (LNA) based probes are examples of a hydrolysis mechanism. In hybridation probes, as Molecular Beacon, fluorescence is avoided because of the hairpin structure of the probe, which maintains together the two molecules in the extremes, and it is released when the probe hybridizes with the complementary region of the DNA target (Gašparič et al. 2010). Probe- based technology reduces interferences due to the formation of primer dimers and nonspecific products (Brzezinski 2006). However, very few studies have been published addressing a practical comparison among different chemistries for allergen detection. Primer based chemistry, such as Lux, Plexor and AmpliFluor, is less common than intercalant dyes and probe-based assays and application for tree nut allergen detection by real-time PCR has not been described (Navarro et al. 2015).

The selection of the specific target is an essential part of a PCR experiment design, because specificity, sensitivity and potential of quantification of the method are directly affected by the target. The targeted DNA sequences are not necessarily located in the genes encoding for the allergenic protein, and thus the analysis detects genomic DNA of the offending ingredient, but does not necessarily indicate the presence of the protein that is responsible for the allergic response (Kirsch et al. 2009). Some researchers have used multi copy genes such as mitochondrial, chloroplast or

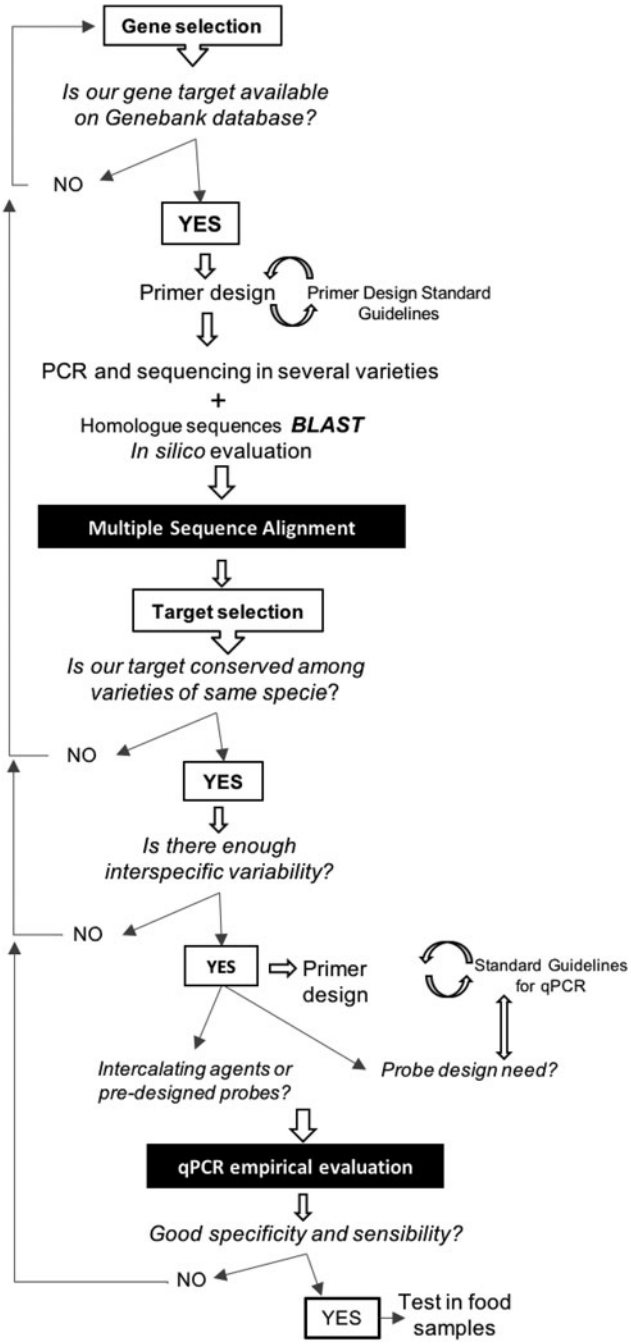

Figure 2. Workflow for the selection of suitable markers for the development of DNA detection methods by real time PCR assays.

repetitive sequences as ITS, to detect an allergenic ingredient in food, in these approach the target have a high number of copies per genome and it is possible to achieve high sensitivity (Demmel et al. 2008; López-Calleja et al. 2013). Other researchers have published detection methods using allergen coding sequences as a target, as Ara h 2 in peanut (Hird et al. 2003), Cor a 9, 11 and 13 in hazelnut (Iniesto et al. 2013), Jug r 1 and 3 in walnut (Linacero et al. 2016), Pis v 1 (Sanchiz et al. 2017) or Ana o 1 in cashew (Brzezinski 2006; Piknová and Kuchta 2007; Sanchiz et al. 2018b) among others. López–Calleja et al., using as target the ITS1 region, have reported a higher sensitivity (0,1 mg/kg almond, cashew, macadamia, pecan, pistachio and walnut) than that

Table 5. Advantages and disadvantages of PCR-based methods and emerging technologies and application to tree nut allergen detection.

Method	Advantages	Disadvantages	Tree nut detection	References
End-point PCR	Cost saving, simple use	Qualitative, sensitivity	Hazelnut Walnut Pistachio	Herman et al 2003; Holzhauser et al 2000 Yano et al. 2007 Barbieri and Frigeri 2006
Multiplex-PCR	Time and cost saving, less cross-contamination	Sensitivity, availability of fluorophores, assay design	Brazil nut, pecan Peanut, hazelnut	Hubalkova and Rencova2011 Renčová et al. 2014
Real-time PCR (qPCR)	Quantitative potential, specificity, sensitivity, processed material use	Cost (*vs end-point PCR*), standardized material requirement	*See* Table 3	*See* Table 3
Multiplex qPCR	Time and cost saving, less cross-contamination	Sensitivity, availability of fluorophores, assay design	*See* Table 3	*See* Table 3
Nested-qPCR	Sensitivity, specificity	Time, assay design (*vs PCR*)	*See* Table 3	*See* Table 3
PCR-ELISA	Sensitivity, specificity	Semi-quantitative	Hazelnut	Tortajada-Genaro et al. 2012; Holzhauser et al. 2002
PCR-HRM	Time saving, specificity, multi-sample analysis	Sensitivity (*vs qPCR i.e.*)	Almond Hazelnut	Costa et al. 2012 Madesis et al. 2013
LPA/MLPA	Cost reduction (*vs multiplex*), quantitative potential, specificity	Assay design	Cashew, pistachio, hazelnut, almond, walnut, Brazil nut, Macadamia Hazelnut	Ehlert et al. 2009 Mustorp et al. 2011
PNA array	Sensitivity, high affinity, resistance to salt presence	PCR needed, cost	Hazelnut-peanut	Rossi et al. 2006
Droplet Digital PCR	Resistance to PCR inhibitors, quantitative, independent of standard reference materials, specificity, sensitivity	Cost	N.A.	N.A.
Optical thin-film genosensor	Sensitivity, specificity, time saving, *in situ* analysis	Equipment required, susceptibility to turbidity interferences	Soybean, wheat, peanut, cashew nut, shrimp, fish, beef and chicken	Wang et al. 2011
Electrochemical genosensor	Time saving, cost saving, sensitivity, specificity, simple	High buffered solution interference	Hazelnut	Montiel et al. 2017

N.A. = not applied.

indicated by other researchers who have used genes encoding allergenic proteins as target.

However, allergen-coding sequences are good candidates because are present in a constant and low copy number in the species genome, thus the use of these kind of genes is recommended for quantification purposes (Prado et al. 2016). Moreover these targets are usually more specific and amplify only DNA from the problem species. To design specific primer pairs and to amplify the target genes only in the species of interest, Multiple Sequence Alignments (MSA) analysis are used to detect conserved sequences in all the varieties of the problem species and variable for others related species (Figure 2). This methodology allows to develop primers for the specific detection of an ingredient in processed food increasing the specifity of primers and probes and reducing false negatives due to inter-variety polymorphisms. To carry out the MSA analysis sequences obtained in the lab and/or in silico, from GeneBank, data could be employed (D'Andrea et al. 2011).

Amplicon size also plays an essential role in DNA detection by PCR, especially taking into account the effect of processing on detectability; the smaller is the amplicon, better sensitivity and reaction performance may be achieved. Amplicons below 150 bp are recommended for specific detection of DNA sequences in processed products, always without compromising specificity (Hird et al. 2003; Rodríguez-Lázaro and Hernández 2013).

The efficiency of the real-time PCR must be in accordance to European Network of GMO Laboratories, (2015) acceptance criteria: the average value of the slope of the standard curves should be in the range of $-3.1 \leq$ slope ≤ -3.6, corresponding to amplification efficiencies of 110% to 90% and the individual values of R^2 of the standard curves should be $\geq$0.98 (Bustin et al. 2009, 2010). The sensitivity of the method is analyzed as the limit of detection LOD defined (European Network of GMO Laboratories, 2015) as the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified. Most of the LOD data presented in Table 3 refer to the amount of sample detected in a model food or a binary mixture as mg/kg. In some cases the authors indicate as LOD the lowest amount of DNA detected (Pafundo et al. 2010).

Methods based on real time PCR might provide reliable and accurate estimation of the DNA target from a food ingredient, allowing the compliance with labeling. The limit of quantification (LOQ) is defined as the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of trueness and precision (European Network of GMO Laboratories, 2015). These quantitative analysis is not really performed in most of the reports (Table 3). To achieve this objective is also essential to take into account the food matrix effect (Villa et al. 2018). Several authors have included in their reports calibration curves made from binary mixtures of known amounts of the target ingredient in specific raw food matrices, as wheat flour, peanut or rice flour (Table 3). More processed matrices have been also used, spiked with different amount of the allergenic tree nut: cookies, bread, pesto or sausages, among others (Costa et al. 2013a; López-Calleja et al. 2014a, b). These reports demonstrated that food matrix composition influences on real-time PCR performance, as well as the thermal processing grade in the mixtures used for allergen detection/quantification (Costa et al. 2017b; Villa et al.

2018; Sanchiz et al. 2018b). Most of the experts have defended that accurate quantification of an allergen in a food product would require a unique standard curve, according to their composition and processing conditions, to take into account different factors affecting DNA quality and yield (as DNA degradation) and and/or the presence of other DNAs in food matrices which might interfere with the PCR reaction. This would be unpractical and it would difficult the application of a normalized real-time PCR system to quantify tree nut in food products (López-Calleja et al. 2014a). Thus, in the case of the detection of allergens, the most important event is to detect the presence or absence.

Nested PCR is a two-step procedure: first amplifying the target sequence with the outer primers and then using the product as template for amplification with the inner primers. There are still some disadvantages, such as an increased risk of carry-over cross-contamination, the complexity of manipulation compared to one-step PCR and the amount of time required. Investigators have attempted to develop one-tube or single-tube nested PCR technologies, in which both outer and inner primers are contained in a single tube, the initial PCR cycles are performed at high annealing temperatures, and the later cycles at low annealing temperatures, thus reducing contamination and maintaining high sensitivity. Single-tube nested real-time PCR incorporates a real-time detection system based on the use of fluorescence (Costa et al. 2012a, 2013a, b). The single-tube nested real-time PCR system, has been already applied to hazelnut, almond and walnut detection (Table 3). The method consists of using two sets of primers and a hydrolysis probe specifically designed to target a gene encoding an allergenic protein. With these systems was possible to reduce the limit of detection of the real-time PCR from 100 mg/kg to 50 mg/kg, moreover the authors showed a Dynamic range of quantification (Table 3).

In addition to the conventional singleplex, PCR or real-time PCR reactions have the potential of simultaneously amplifying more than one analyte in a food matrix by using a multiplex format. Due to the number of food allergens, the multiplex detection of different allergenic ingredients is of great interest for control laboratories. Regarding tree nut detection, Table 3 collected some reports in which multiplex approach has been applied. Köppel et al. (2010) developed two tetraplex real-time PCR, detectingd simultaneously peanut, hazelnut, celery, and soy in one multiplex reaction and egg, milk, almond, and sesame in another different reaction, with a LOD of 100 ppm. Pafundo et al. (2010) developed a multiplex PCR assay to detect almond, cashew, walnut simultaneously. This multiplex format has the advantages of saving time, costs and reducing probability of cross-contamination. However, it often decreases sensitivity of the assay, compared to singleplex PCR (Table 5).

5. Other DNA-based techniques

In recent years, several interesting approaches have been developed to detect and, in some cases, quantify DNA targets. Some of these approaches have a promising potential for the detection of allergenic ingredients in food products, including PCR-ELISA, High Resolution Melting (HRM), Ligation-dependent Probe Amplification (LPA) and Peptide Nucleic Acid (PNA) arrays. Table 5 summarized several DNA based methods used for tree nut allergen detection, including main advantages and disadvantages for each one.

Polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA) is an immunodetection method to quantify the PCR product, combining PCR amplification with detection by ELISA. The DNA fragment is directly labeled with biotin or digoxygenin during PCR and is hybridized to an immobilized capture probe (Kirsch et al. 2009; Słowianek and Majak 2011). DNA can be quantified by the color reaction that results from an enzyme-substrate reaction due to the coupling of the protein label with a specific enzyme-labeled antibody. This method, which detects nucleic acid instead of protein, is high specificity and sensitivity and reliable high-throughout for specific detection of target DNA within PCR amplicons but only could be considered semi-quantitative because the quantification is performed at the end of the PCR reaction and not during the exponential growth phase, as in real time PCR. Digoxin-labeled PCR products from hazelnut, peanut, and soybean DNA were detected by hybridization with 5′-biotinylated probes that were immobilized on a streptavidin-modified digital versatile disk surface, providing a LOD of 1 µg/g (Tortajada-Genaro et al. 2012). In a comparative study between PCR-ELISA and protein sandwich ELISA for the detection of hazelnut, similar sensitivity was reported for both approaches and a higher specificity was reported for PCR-ELISA (Holzhauser et al. 2002). When compared to real-time PCR, PCR-ELISA has demonstrated comparable sensitivity and specificity with no requirement for special or expensive equipment (Hadrich et al. 2011).

HRM analysis involves the gradual melting of PCR amplicons with increasing temperature and the detection of subtle fluorescent changes (Costa et al. 2012a). Due to advances in both high resolution instrumentation and saturating fluorescent DNA binding dyes, the development of HRM analysis has been applied to genotyping (Herrmann et al. 2006; Reed et al. 2007), epigenetics (Wojdacz and Dobrovic 2007) and studies in food products (Jaakola et al. 2010; Ganopoulos et al. 2011; Sakaridis et al. 2013), among others. Applications of HRM analysis for the identification of food ingredients have been developed because differentiation down to the genus or even species level is now possible based on the melting temperature (Tm) of specific PCR products (Cheng et al. 2006). HRM analysis has been used in a real-time PCR system with Evagreen to detect the presence of almond in food products; this approach has been shown to be able to discriminate almond from other Prunus fruits and other tree nuts, such as walnut, macadamia nut, and hazelnut (Costa et al. 2012a). The authors reported the specific detection of a relative LOD of 50 mg/kg of almond in walnut. Madesis et al. (2012) have also used HRM that was combined with DNA barcode (Bar-HRM); for the differentiation of legume species via the use of universal chloroplast bar-coding regions, this approach allowed

the detection of lupine DNA in soybean flour with a detection limit of 1%. Hazelnuts have also been quantitatively identified by Bar-HRM in processed food products, and a detection limit of 0.01% was confirmed (Madesis et al. 2013).

As it was previously mentioned, the multiplex detection of different allergenic ingredients is of great interest for control laboratories. Several real-time PCR based methods have been developed in recent years for the simultaneous detection of two or more allergenic ingredients (Rossi et al. 2006; Schöringhumer et al. 2009; Köppel et al. 2010; Pafundo et al. 2010, 2011, Fuchs et al. 2010; Galan et al. 2011; Janská et al. 2011; Köppel et al. 2012). Multiplex PCR methods have several advantages, thought they frequently have lower sensitivity than simplex PCR (Rossi et al. 2006), and might require a careful optimization of the primer concentration to avoid the preferential amplification of some of the targets (Pafundo et al. 2010). Moreover, this technique is limited by the reduced number of fluorophores to determine several genes simultaneously (Tortajada-Genaro et al. 2012). Ligation-dependent Probe Amplification (LPA) has been suggested as a good alternative to overcome some of the limitations of multiplex real time PCR. LPA is based on the amplification of products that result from the ligation of bipartite specific hybridization probes (Ehlert et al. 2009) and uses a single pair of PCR primers to amplify several target sequences. One probe oligonucleotide contains the sequence that is recognized by the forward primer, and the other probe contains the sequence that is recognized by the reverse primer. Only when both probe oligonucleotides are hybridized to their respective targets, can these probes be ligated into a complete probe; therefore, this technique yields an amplification product of unique size (Schouten et al. 2002) that can be discriminated by capillary electrophoresis. Since all PCR fragments are amplified by the same primer set, a competitive PCR is created where all the fragments are amplified with the same efficiency (Mustorp et al. 2011). LPA has been used for the detection of 10 different allergenic ingredients (peanut, cashew nut, pecan, pistachio, hazelnut, sesame, macadamia nut, almond, walnut and Brazil nut) in the low mg kg^{-1} range; when tested in different matrices (cookies, pesto and chocolates), this method allowed an LOD of 5 mg·kg^{-1} for single allergens (Ehlert et al. 2009). Taking advantage of the fact that all fragments are amplified with the same efficiency and, therefore, that the initial ratios of amplicons are conserved throughout the PCR, Mustorp et al. provided quantitative results using LPA. By comparing signals with an internal positive control for eight allergenic ingredients, the LOD varied from 5 to 400 genes copies, depending on the allergen (Mustorp et al. 2011). To ease multiplex detection, several arrays have also been used for the detection of PCR products and applied to the detection of allergenic ingredients in food products.

Most of the disadvantages of DNA-based methodologies compared to real-time PCR are related to a possible lack of sensitivity, as in end-point PCR or LPA/MLPA. Until now, real-time PCR has been the better methodology for the quantitative analysis of specific DNA in a sample. Also, real-time PCR is less prone to contamination and is suitable for automation. Numerous assays based on real-time PCR have been developed and have proved be effective for the detection of a wide range of traces of food allergens in raw materials and processed foods (Table 5).

As explained before, protein-based methods are extensively applied in food industries and control agencies, as commercial ELISA kits. The commercially available real-time PCR kits are less common and normally provide qualitative information (Costa et al. 2014). The time per analysis and sensitivities reported for these kits are similar to ELISA, with the advantage of not presenting known cross-reactivities. Many publications provide evidence of robust DNA based detection and good correlation between DNA and protein based analysis of allergenic foods. Platteau et al. (2011b) demonstrated that food processing has an impact on hazelnut detection in cookies and cookie ingredients using real-time PCR as well as ELISA. According to the authors, both methods lacked robustness with regard to food processing and hazelnut detection in foodstuff highlighting the need for adequate reference materials. Costa et al. (2014) evaluated the performance of several methodologies (LC-MS/MS, real-time PCR and ELISA) regarding the detection of hazelnut in model chocolates. The authors concluded that all the performed methods were appropriate for the identification of hazelnut in complex foods such as chocolates.

6. Emerging technologies

Reliable and rapid food allergen test methods are needed by both control laboratories and by the food industry to ensure the health of consumers, to easily determine whether a food product has been subjected to cross-contamination and, simultaneously, to identify how and when this cross-contamination occurred to establish the proper corrective measures. Rapid immunoanalytical tests for the determination of allergenic food proteins have been recently reviewed (Schubert-Ullrich et al. 2009); therefore, we will focus on recent advances for the detection of allergenic ingredients and on the use of nanomaterials for this application. Some of these emerging technologies, as digital PCR or genosensors, could be promissing for tree nut allergen detection (Table 5).

Digital PCR (dPCR) is based on the use of limiting dilutions, PCR and Poisson statistics to quantify the absolute number of targets that are present in a sample. During dPCR, the PCR mix is distributed across a large number of partitions containing zero, one or more copies of the target nucleic acid. An end-point PCR is performed in each of these partitions and is defined as positive when the PCR product is present or negative when the PCR product is not present (Morisset et al. 2013). dPCR are explored for the quantification of allergenic foods as a good solution to overcome some of the difficulties that have been previously mentioned for establishing a quantitative result with real-time PCR (Corbisier et al. 2010). Morisset et al. (2013) have found that their approach for the quantitative analysis of genetically modified organisms showed comparable

sensitivity, with better repeatability at low range concentrations and a greater tolerance to inhibitors when compared with real-time PCR. In addition, dPCR allows the accurate measurement of DNA copies without the need for a reference calibrator and is not dependent on amplification efficiency (Corbisier et al. 2010). To our knowledge, no dPCR method in the particular area of food allergen detection has been published thus far, but this technique might be valuable for the estimation of allergenic ingredients in foodstuff (Bhat et al. 2011, Morisset et al. (2013). Biosensors are devices employing biological recognition properties for a selective bioanalysis (Kumar 2008), and comprise three components: a biological receptor that is specific to the analyte to be analyzed, a transducer to convert the recognition element into a suitable signal and a reader device with the associated electronics or signal processors that are responsible for the display of the results. Target molecules, such as proteins or DNA, can be immobilized on a sensor chip surface and the binding activity between recognized molecules can be quantitatively monitored (Schubert-Ullrich et al. 2009). The main goal of the research in this field is the development of lab-on-a-chip systems that allow the fast, sensitive and inexpensive analysis of samples, without the cumbersome pre-analysis or preparation of samples, in contrast to the usual analytical techniques. This fact makes biosensors an interesting alternative to be used by small control laboratories or to perform fast in situ analysis by the food industry. Amplification using two multiplex PCR and analysis of the results by a biochip assay including specific capture probes was performed for simultaneous detection of sesame, oat, almond and celery or hazelnut, walnut, lupine, and mustard (Wang et al. 2011a). Absolute and practical LOD was stablished in 0.5 pg and 10 mg/kg of sesame DNA and sesame flours respectivelly. Cross-reactivity among species was not found by the authors, and applicability of the method was confirmed by analyzing commercial samples (Wang et al. 2011a). Similarly, Wang et al. (2011b) developed an optical thin-film microarray method on a silicon-based surface tested for the simultaneous detection of eight food allergenic ingredients from two tetraplex PCR systems for the simultaneous and specific detection of cashew, peanut, wheat and soy or chicken, fish, shrimp and beef. As a reference, authors showed the stabilishement of absolute and practical LOD for cashew DNA and flour (0.5 pg and 10 mg/kg, respectively). In both reports, signals can be observed by the naked eye, without the need of additional instrumentation.

Despite the interesting performances obtained, the demand for sensitive and sequence-specific approaches that do not require laborious or expensive detection technologies has led to DNA biosensing. DNA biosensing methodologies are usually based on the detection of DNA hybridization, and due to the difficulty to detect DNA by only amplifying the signal, sample preamplification by means of PCR is commonly required (Pedrero et al. 2011). This strategy combines the high selectivity of DNA-based methods with the interesting advantages of sensors, as electrochemical ones. Apart for simplifying the process, making analytical procedures friendlier, electrochemical DNA sensors offer multiplexing capabilities and can meet important market demands allowing the fabrication of compact and low cost devices. In this context, screen-printed sensors using magnetic microbeads and previous PCR amplification are particularly attractive because of their excellent sensitivity, reduced time of analysis, minimization of matrix effects, high sample throughput, easy automation with mínimum consumption of the sample and reagents and possibility of achieving inexpensive and decentralized measurements. Recently, Montiel et al. (2017) designed an assay that combined Express end-point PCR and magnetic microbeads technology for the specific and unequivocal detection of hazelnut in less that 2 hours. This sensor involved detection of previously amplified Cor a 9 allergen coding sequence, allowing the identification of hazelnut genomic DNA up to 20 pg and compares advantageously, in terms of sensitivity, simplicity and assay time with conventional electrophoresis gel methodology and other real-time PCR protocols (Montiel et al. 2017).

DNA-probe immobilization is a crutial step and stem loop DNA structures have been proposed as capture probes with better performance than linear probes, regarding the discrimination of mismatches. An electrochemical DNA sensor based on a stem-loop probe dual and labeled with a tiol group on 5′ and biotin in 3′ end was designed for the detection of a fragment of the Ara h 1 gene for peanut detection (Sun et al. 2012). The stem-loop probe was “closed” when the target was absent, and “open” after hybridization with the target, promoting electron- transfer efficiency changes due to the detachment of biotin from the electrode. With a calculated LOD of 0.35 fM of DNA target in standard solution hybridized on the sensor, and without the need of previous target amplification, the proposed strategy was applied to detect Ara h 1 in DNA extracts of peanut milk beverage. Selectivity of the assay, by hybridation of the sensor with other DNA sequences, was succesfully evaluated (Sun et al. 2012). Authors who developed biosensors for allergen detection have expressed LOD as concentration in M. It is an absolute LOD, calculated from concentration curves of DNA hybrizated in the sensor, using a standard solution, not a food matrix, so it is not comparable to practical LOD expressed as mg/kg by ELISA or real-time PCR.Likewise, the appearance of more specific and stable ligands might greatly contribute to the development of more reliable detection methods and biosensors.

In this regard, aptamers, nucleic acids with appropriate secondary structures, single stranded (ssDNA) or RNA oligonucleotides that function as ligands, are emerging as a new analytical strategy which can be coupled with different transduction systems (Amaya-González et al. 2013). Aptamers can bind diverse targets, such as small ions, proteins or even cells. Aptamers possess a high affinity for their targets due to their capability of folding upon binding with their target molecule (Song et al. 2008). Aptamers are selected in vitro from large populations of random sequences through a combinatorial strategy called SELEX (systematic evolution of ligands by exponential enrichment, (Tuerk

and Gold 1990). Aptamers might be a relevant alternative to antibodies in bioanalytical applications (Iliuk et al. 2011). Much work remains to be performed to integrate aptamers into biosensors for food control and tree nut allergen detection. In this sense, an aptamer-based biosensor has been developed to specifically bind Ara h 1, both in buffer or food matrix, demonstrating a relevant potential for peanut detection (Tran et al. 2013). Nadal et al. (2012) demonstrated the functionality of a selected aptamer which binds specifically to Lup an 1 allergen from lupin, achieving a LOD of 153 nM. This system was capable to specifically bind to mentioned allergen distinguishing it from other possible proteins present in flour (Nadal et al. 2012).

7. Conclusions and future trends

This review shows how qPCR has been widely utilized to detect tree nut allergens in dairy processed food products because of its advantages over protein-based methods. qPCR is competitive in terms of sensitivity, costs, and speed of execution when compared with classical immulogical methods (ELISA).

PCR-based methods are no longer more expensive than immune-based techniques and they have some key advantages over immunochemical methods. Food processing can affect the conformation and solubility of proteins andharsh extraction methods cannot typically be used since they could further affect antibody-binding epitopes. With DNA however, harsher extraction buffers can be used without affecting the detection of the target DNA. Amounts of DNA also tend to be more stable than protein levels, which can vary between various species or varieties. An additional advantage is that PCR-based tests are available for detection of DNA from a number of allergenic sources for which ELISA methods may not be available. Therefore, with the stability of DNA, qPCR becomes an elective platform in assessing the allergenic risk of food products. Recent advances, as ddPCR and HRM, will give to qPCR a step forward in the accuracy and speed of analysis. With next generation equipment, there will be the possibility of using the platform on-line, more successfully meeting the needs of industry.

Abbreviations

Ct	cycle threshold
CTAB	hexadecyltrimethylammonium bromide
LTP	lipid trasfer protein
HHP	high hydrostatic pressure

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