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REVIEW

# Food authentication by PCR-based methods

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**Abstract** Food authenticity is presently a subject of great concern to food authorities, as the incorrect labelling of foodstuffs can represent a commercial fraud. The implication of misleading labelling can be much more important concerning the presence of potentially allergenic foods. The need to support food labelling has provided the development of analytical techniques for the analysis of food ingredients. In the last years, several methods based on polymerase chain reaction (PCR) have been proposed as useful means for identifying species of origin in foods, as well as food allergens and genetically modified organisms (GMO), due to their high specificity and sensitivity, as well as rapid processing time and low cost. This work intends to provide an updated and extensive overview on the PCR-based methods for food authentication, including also methods for allergens and GMO the detection in foods.

**Keywords** Food authenticity · PCR · Meat products · Seafood · Dairy products · Allergens · GMO

## Introduction

The increased awareness of consumers regarding the composition of foods has resulted in the need to verify the labelling statements. The incorrect labelling of foods represents a commercial fraud, considering the consumer acquisition. It is very important to establish that species of high commercial value declared are not substitute, partial or entirely, by other lower value species. The misleading labelling might also have negative implications concerning health, especially for sensitive consumers to nondeclared potential allergens. The food allergies are considered an emergent public health problem, especially in developed countries. Recently, the *Codex Alimentarius* FAO/World Health Organization (WHO) and the European Commission proposed a list of allergens based on the prevalence and severity of allergens, which should be labelled in prepacked foods. The list includes 12 groups of ingredients potentially allergenic from which milk, eggs, fish, crustaceous, peanuts, soybean, walnuts, whey and other cereals with gluten are emphasised [1].

The information given to consumers is also essential for them choosing certain foods over others. That choice might be the reflection of lifestyles, such as vegetarianism, or religious practices, such as Jews and Muslims, where pork meat should be absent. The recent occurrence of several food crises has emphasised food safety and protection of consumer's health as main objectives for the food labelling legislation [2]. Thus, when considering all the referred concerns, the need to verify the labelling statements is a crucial aspect for consumers, food industries and food authorities.

The analytical methods used for species identification and authenticity of foods rely mainly on protein and DNA analysis. The protein-based methods include immunological assays [3, 4], electrophoretical and chromatographic techniques [5–7]. More recently, DNA molecules have been the target compounds for species identification due to the high stability compared with the proteins, and also to their presence in most biological tissues, making them the molecules of choice for differentiation and identification of

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components in foods, and a good alternative to protein analysis [8, 9]. Most DNA-based methods for species identification in foods consist on the highly specific amplification of one or more DNA fragments by means of polymerase chain reaction (PCR). This technique presents a high potential due to its fastness, simplicity, sensibility and specificity [9, 11].

The PCR amplification is based on the hybridisation of specific oligonucleotides (primers) and synthesis, in vitro, of millions of copies flanked by those primers [12]. The specific amplification of one fragment followed by agarose gel electrophoresis for fragment size verification is the simplest PCR strategy to evaluate the presence of a species. Additional confirmation methods and/or analysis of fragments can be done by the digestion of fragments with restriction endonucleases (PCR-RFLP), sequencing techniques of fragments, simultaneous amplification of two or more fragments with different primer pairs (multiplex PCR), analysis of single strand conformation polymorphisms (PCR-SSCP), which is based on the electrophoretical mobility of the amplified fragments in their single strand and folded conformation [9]. Other strategies used in the identification of species in foods include the analysis of ramdom amplified polymorphic DNA (RAPD), which involves the use of single and short arbitrary primers, and the analysis of single sequence repeats (SSR), also known as microsatellites.

A crucial aspect in food analysis, as in the case of genetically modified organisms (GMO) or allergens, is the quantification, which is imposed by the legislation requirements. The lack of quantitative information obtained with end point PCR, due to an inconstant efficiency along the amplification cycles, led to the development of two quantitative detection strategies in foods: quantitative competitive PCR (QC-PCR) [13] and real-time PCR [9]. The possibility of using specific probes or labelled primers by real-time PCR allows the simultaneous detection and confirmation of fragments, increasing the reliability of the technique and the number of PCR applications to food analysis [14, 15].

The need for accurate and reliable methods for species identification in foods has increased steadily in the past few years [16]. The PCR techniques have been successfully applied in the detection of animal origin in meat products [17], dairy products [18] and fish products [19]. The number of applications of PCR techniques in products of plant origin is huge, especially if we account with those related to the GMO detection [8–11, 15, 20, 21].

The purpose of this review paper is to describe an updated overview of the applications of PCR technique in the identification of species of origin in foods for authenticity assessment.

## Foods of animal origin

### Meat products

A frequent adulteration of meat products is the addition of pork (*Sus scrofa*) to beef (*Bos taurus*) products for economic gain. Undeclared pork is an undesirable contaminant regarding religious practices as well as dietary and health reasons, such as allergies or the introduction of food-borne microorganisms. The presence of pork DNA was determined by species-specific PCR primers of porcine growth hormone gene, enabling the detection of pork in fresh or heated mixtures of pork in beef at levels below 2% (w/w) [22]. Detection of pork, beef, buffalo, ewe, goat, horse, chicken and turkey in marinated, processed and fermented products by amplification of mitochondrial cytochrome *b* gene and analysis of restriction fragments (PCR-RFLP) was described by Meyer et al. [23]. This method enabled the detection of pork in meat mixtures of pork and beef at levels below 1% (w/w). Specific detection of pork was also obtained by amplification of *D-loop* mitochondrial DNA from meat and fat in meat mixtures, including those dry-cured and heated by cooking [24]. The PCR amplification of pork mitochondrial genes (12*S* rRNA and cytochrome *b*) was successfully applied in the identification of pork derivatives in different types of food products, proving to be a reliable and suitable technique in routine food analysis for halal certification [25, 26].

Calvo et al. [27] developed and evaluated a semi-quantitative PCR technique to detect pork in ground beef and pâté targeting a repetitive DNA element (SINE). The results allowed the specific detection of 0.005% (w/w) of pork in beef (raw and heated) and the semi-quantification of pork contamination up to 1%. The application of the referred technique to commercial pâtés evidenced the fraudulent additions of pork meat. The quantitative assessment of pork species in meat mixtures was achieved by QC-PCR [28] and real-time PCR with TaqMan probes [29].

Game meat and derived products are often targets for fraudulent labelling due to the high commercial value associated to these products. By means of a PCR-RFLP technique with two endonucleases, Wolf et al. [30] were able to discriminate 25 game species. Brodmann et al. [31] noted that the lack of reference materials for game meat could be solved by sequencing PCR fragments and comparison with data base sequences for the highest percentage of correspondence. Fajardo et al. [32] developed a PCR-RFLP method for the identification of meats from red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), cattle (*Bos Taurus*), sheep (*Ovis aries*), and goat (*Capra hircus*). Another work from the same authors [33] reported the identification of meats from chamois (*Rupicara rupicara*), Pyrenean ibex (*Capra*

*pyrenaica*) and mouflon (*Ovis ammon*) by species-specific amplification of mitochondrial *D-loop* sequences, with satisfactory application to pasteurised and sterilised meats.

Fois gras is a traditional luxury food prepared from the liver of geese or ducks, which has been linked to the French gastronomy. Fraudulent labelling practices exist because goose foie gras is most appreciated by consumers and also the most expensive. A PCR technique was used for the specific identification of goose (*Anser anser*) and mule duck (*Anas platyhynchos* × *Cairina moschata*) fois gras [34, 35]. Identification of chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*) and pork (*Sus scrofa*) in goose and mule duck foie gras is also described by species-specific PCR. Quantification of mule duck in goose foie gras in a range of 1–25% (w/w) was possible to obtain by real-time PCR with TaqMan probes [38].

A quick and simple method for the identification of six meat species (cattle, pork, chicken, sheep, goat and horse) in raw and cooked meats was developed by Matsunaga et al. [39] by means of multiplex PCR targeting the mitochondrial cytochrome *b* gene. The use of PCR fingerprinting techniques, such as RAPD, allowed the discrimination of several common meat species [40, 41]. Rastogi et al. [41] proved that mitochondrial DNA-based markers are more suitable to discriminate meat species than the nuclear markers. Among PCR fingerprinting approaches, RAPD was found to be more discriminatory and accurate for meat species [41]. The PCR-RFLP technique was applied with success to explore the incidence of incorrect labelling in food products containing one or more meat species [17]. The undeclared presence of turkey and the absence of declared species of high commercial value such as beef or roe-deer were the detected frauds. A strategy for molecular species detection in meat and meat products by PCR-RFLP and DNA sequencing using mitochondrial and chromosomal genetic sequences was developed by Maede [42]. Species-specific real-time PCR assays were successfully used for detection of beef, pork, lamb, chicken and turkey with levels of detection below 0.1% (w/w) [43]. The application of several real-time PCR systems for identification and quantification of different species in complex DNA mixtures enabled levels of detection lower than 0.80 pg of DNA [44, 45]. An oligonucleotide microarray hybridisation analysis of PCR products was applied to identify different animal species in meat and cheese samples [46]. The species-specific probes identified unequivocally cattle, pig, chicken, turkey, sheep and goat and were able to detect simultaneous up to four different species in mixtures.

Recent outbreaks of bovine spongiform encephalopathy (BSE) in cattle and the potential for transferring BSE to humans in the form of variant Cruetzfeld Jacobs Disease have increased the call for traceability of meat, meat products and feedstuffs [47]. Thus, to prevent commercial frauds and for food safety purposes there is the need of methods to detect beef in food bovine derived materials in feed. Calvo et al. [48] focused their work on specific PCR amplification of a repetitive DNA element for the identification of bovine contamination, obtaining levels of detection of 0.005% (w/w) of raw beef in pork and 1% of beef in cooked meat mixtures. A species-specific PCR technique targeting the mitochondrial cytochrome *b* gene of bovine DNA was successfully applied to processed meats with a sensitivity of 0.025% (w/w) [49]. Laube et al. [50] developed two TaqMan real-time PCR systems able to distinguish between beef and pork in a total of eighteen animal species and with sensitivities lower than 0.1% (w/w) for both species. The detection of bovine material in gelatine was developed by Tasara et al. [51] using conventional and real-time PCR approaches. The inclusion of bovine gelatine in pork or fish gelatine could be detected with a sensitivity of 0.1% (w/w) by species-specific PCR and 0.001% by real-time PCR.

As alternative to microscopic analysis, currently employed to identify the contents of animal feed, several PCR-based assays have been proposed. Species-specific PCR primers have been successfully applied in the detection of animal material in feedstuffs derived from cow, sheep, pork, chicken and goat [52–59]. Quantitative detection of animal species in feeds has also been attemped based on QC-PCR [60] and real-time PCR [61–63]. A collaborative trial indicated that the real-time PCR could reliably be used for detection of BSE risk material in meat and meat products [64].

The application of PCR-based techniques in the authenticity assessment of meat products are resumed in Table 1.

## Seafood

The change of consumer's attitudes towards health and nutrition has increased the consumption of seafood and derived products. Some of the world's fish catch is sold unprocessed, which enables the easy the identification of species through rigorous inspection, by applying taxonomic classification methods based on morphological characteristics, such as the skin pigmentation, shape, size and appearance, Actually, most fish species are available commercially after the removal of some external features as fresh (eviscerated, beheaded, skinned, filleted, etc.) and processed products (marinated, salted, smoked, caned, frozen, etc.), which makes the identification of species a difficult task. Even when the morphological characteristics are intact, the similarities between very closely related species make their identification rather difficult and only a taxonomist can perform it correctly. On the other hand, the EU labelling regulations [65, 66] specify that the commercial and scientific names should be included on the label of

**Table 1** Summary of PCR-based methods applied in the authentication of meat products

| Food product | Species | Technique | Target gene | Limit of detection[a] | References |
|---|---|---|---|---|---|
| Bovine meat products (marinated, processed, fermented, dry-cured, halal) | Pork | Species-specific PCR | Growth hormone | 2% | [22] |
| | | PCR-RFLP | Cytochrome *b* | <1% | [23] |
| | | PCR-RFLP | Cytochrome *b* | NR | [25] |
| | | PCR-RFLP | *D-loop* | 5% | [24] |
| | | Species-specific PCR | 12*S* rRNA | NR | [26] |
| | | Species-specific PCR | SINE | 0.005% | [27] |
| | | QC-PCR/PCR-RFLP | Growth hormone | 0.1%, 100 pg DNA | [28] |
| | | Real-time PCR | 12*S* rRNA | 0.1%, 0.01 ng DNA | [29] |
| Game meat | 25 game species | PCR-RFLP | Cytochrome *b* | NR | [30] |
| | Cervidae species | PCR-RFLP/sequencing | Cytochrome *b* | NR | [31] |
| | Red deer, fallow deer, roe deer, cattle, sheep, goat | PCR-RFLP | 12*S* rRNA | NR | [32] |
| | Chamois, Pyrenean ibex, mouflon | PCR-RFLP | *D-loop* | 0.1% | [33] |
| Fois gras | Goose, mule duck | Multiplex PCR | 5*S* rDNA | NR | [34] |
| | Goose, mule duck | Species-specific PCR | α-actin | 1% | [35] |
| | Chicken, pork | Species-specific PCR | α-actin | 0.1% | [36] |
| | Goose, mule duck, chicken, turkey, pork | Species-specific PCR | 12*S* rRNA | 1% | [37] |
| | Mule duck, goose | Real-time PCR | 12*S* rRNA | 0.01% | [38] |
| Cooked meat products | Cattle, pork, chicken, sheep, goat, horse | Multiplex PCR | Cytochrome *b* | 0.25 ng DNA | [39] |
| | Chicken, beef, pork, turkey, quail, duck, follow deer, red, deer, roe deer, wild boar | PCR-RFLP | Cytochrome *b* | NR | [17] |
| | Pork, beef, lamb, chicken, turkey | RAPD/fingerprinting | – | NR | [40] |
| | Buffalo, cow, chicken, pork, goat, frog, fish, snake | PCR sequencing/RAPD/ fingerprinting | 16*S* rDNA/ND4/actin | NR | [41] |
| | Cattle, buffalo, red deer, sheep, pork, horse, chicken, turkey | PCR-RFLP/sequencing | Cytochrome *b*/ growth hormone | 0.1–1% | [42] |
| | Beef, lamb, pork, chicken, turkey | Real-time PCR | Cytochrome *b* | 0.1–0.5% | [43] |
| | Cow, pork, lamb, chicken, turkey, ostrich | Real-time PCR | 18*S* rRNA | 0.03–0.80 ng DNA | [44] |
| | Cow, pork, horse, wallaroo | Multiplex/Real-time PCR | Cytochrome *b*/ND6 | < 1%, < 0.4 ng DNA | [45] |
| | Cow, pork, chicken, turkey, sheep and goat | PCR oligonucleotide microarray | Cytochrome *b* | 0.1% | [46] |
| | Cow | Species-specific PCR | Microsatellite DNA | 0.005–1% | [48] |
| | Cow | Species-specific PCR | Cytochrome *b* | 0.025% | [49] |
| | Cow, pork | Real-time PCR | Phosphodiesterase/ ryanodin/myostatin | <0.1% | [50] |
| Ground beef | Cow individuals | SSR | Microsatellite DNA | NR | [47] |
| Gelatine | Cow | Species-specific PCR/ real-time PCR | ATPase subunit 8 | 0.001–0.1% | [51] |

*NR* Not reported

[a] Values in percentage are expressed on a weight by weight basis

seafood products. These facts demand available methodologies able to detect species in processed seafood. DNA-based methods have proved to be alternative to protein-based methods and became the methods of preference for the analysis of a wide range of seafood products [67]. This section describes the applications of PCR-based methods in the identification of seafood, including molluscs, which are resumed in Table 2.

## Fish and fish products

PCR-based methods for fish species identification include sequencing amplified genomic DNA [68, 69], species-specific PCR primers [70], PCR-RFLP [68, 71–74], PCR-SSCP [75–77] and RAPD [78]. Most DNA-based methods for fish species identification consist in the amplification of mitochondrial DNA, as observed in other food matrices.

**Table 2** Summary of PCR-based methods applied in the authentication of seafood products

| Food product | Species | Technique | Target gene | References |
|---|---|---|---|---|
| Raw and processed (smoked, heat-treated, marinated, canned, pastry) | Salmonids | PCR-RFLP | Cytochrome *b* | [72, 81] |
| | | PCR-SSCP | Cytochrome *b*/parvalbumin/ growth hormone | [77] |
| Raw and frozen | Flatfish (sole, Greenland halibut, megrim, flounder, turbot) | Species-specific PCR | 5*S* rDNA | [70] |
| | | PCR-RFLP | 12*S* rRNA | [82, 83] |
| Raw and heat-treated | *Serranidae* (Nile perch, grouper, wreck fish) | PCR-RFLP | 12*S* rRNA | [68] |
| | | PCR-RFLP | α-actin | [71] |
| | | PCR-SSCP | 12*S* rRNA | [75] |
| | | RAPD | | [78] |
| | | PCR-ELISA | 5*S* rDNA | [84] |
| | | Multiplex PCR/real-time PCR | 16*S* rRNA | [85] |
| Raw and hot-smoked, canned | Eels | PCR-RFLP/PCR-SSCP | Cytochrome *b* | [76] |
| Raw, frozen, salted and heat-treated | Gadoids | PCR-RFLP | Cytochrome *b* | [69] |
| | | PCR-RFLP/PCR-SSCP/DGGE | Cytochrome *b* | [19] |
| | | PCR-RFLP | 16*S* rRNA | [86] |
| | | PCR-sequencing/RFLP | Mitochondrial control region | [73] |
| | | Real-time PCR | Transferrin | [87] |
| Raw, frozen and canned | Tuna | PCR-RFLP/PCR sequencing | Cytochrome *b* | [88, 89] |
| | | PCR-SSCP | Cytochrome *b* | [90] |
| | | Real-time PCR | 16*S* rRNA | [91] |
| Raw and canned | Sardines | PCR-RFLP/sequencing | Cytochrome *b* | [92] |
| Caviar | Sturgeon | PCR-RFLP/sequencing | Cytochrome *b* | [93] |
| Raw and frozen | Clams | PCR-RFLP | A-actin | [94] |
| | | PCR-SSCP | A-actin | [95] |
| Raw | Mussels | RAPD/species-specific PCR/ sequencing | *ITS* | [96] |
| | | PCR-RFLP/FINS/Multiplex PCR | Adhesive protein/ 18*S* rDNA/*ITS1* | [97] |
| Raw, rings and canned | Cephalopods | PCR-RFLP/FINS | Cytochrome *b* | [98] |

The cytochrome *b* has been most often used for species identification, although other gene fragments have also been used [79].

Wolf et al. [74] described a simple method based on PCR-RFLP technique for the differentiation of 23 species of fish, such as salmon, cod, tuna and eel. However, when considering the differentiation of one particular species in a mixture of species, those authors recommended the use of species-specific PCR primers.

The identification of ten species of white fishes, such as the Atlantic cod, the European hake, haddock and whiting was succeeded by PCR-RFLP and exact confirmation of size fragments by lab-on-a-chip capillary electrophoresis [80]. The method was applied to a range of products and subjected to an interlaboratory study, whose results considered it a qualitative test for screening raw, frozen and lightly processed samples for the presence or absence of fish species, without the need of reference materials.

Russel et al. [81] developed a PCR-RFLP technique for the identification of salmon species based on the amplification of a region of the cytochrom *b* mitochondrial gene. The restriction pattern obtained was resolved by means of polyacrylamide gel electrophoresis (PAGE), allowing the discrimination of ten salmon species. The method was validated in a collaborative study and applied to a wide range of commercial food product containing salmon [72]. In almost all cases the salmon species declared was confirmed, although a trout species was detected in one product declaring only the presence of salmon. The differentiation of ten salmonid fish species belonging to the genera *Salmo*, *Oncorhynchus* and *Salvelinus* was achieved by PCR-SSCP of mitochondrial and nuclear genes followed by analysis in PAGE [77]. The method was successfully applied in the identification of raw or cold smoked salmon, as well as in salmon roe.

The differentiation between close species of flatfish, such as the sole (*Solea solea*) and the Greenland halibut (*Reinhardtius hippoglossoides*), allows the detection of the fraudulent substitution of the first species by the second, which has lower commercial value and is less appreciated.

The distinction of the two species was performed by species-specific PCR targeting a nuclear gene [53] and by PCR-RFLP of a mitochondrial gene [82]. The identification of five species of flatfish of Pleuronectiformes order (*Lepidorhombus whiffiagonis*, *Platichthys flesus*, *Reinhardtius hippoglossoides*, *Scophthalmus maximus* and *Solea solea*) was also achieved by PCR-RFLP of a mitochondrial gene [83].

Nile perch (*Lates niloticus*) fillets are many times labelled and marketed either as grouper (*Epinephelus guaza*) or as wreck fish (*Polyprion americanus*) due to the higher popularity and quality of the two latter species. Additionally, grouper and wreck fish are closely related species that may be misidentified in market and are commonly sold as grouper, which is more in demand by consumers. Thus, several methods have been proposed for the distinction of these three species. The PCR amplification of a sequence of α-actin gene followed by fragment digestion with two restriction enzymes allowed the identification of wreck fish and Nile perch [71]. The differentiation of the three species was achieved by PCR-RFLP [68], PCR-SSCP and separation by PAGE [75] and RAPD [78]. A semi-quantitative assay using a combined PCR-ELISA technique was successfully applied in the detection of Nile perch in mixtures of fish species with a detection level of 1% (w/w) [84]. Trotta et al. [85] used two simple strategies for the identification of grouper species (*Epinephelus* and *Mycteroperca*) and their most common substitutes (Nile perch and wreck fish) by multiplex PCR and real-time PCR.

The eel species *Anguilla anguila*, *A. rostrata*, *A. japonica* and *A. australis* are the most important commercially from the genus *Anguilla*. In Europe, the species *A. anguilla* is preferred for production of hot-smoked eel (e.g. in Germany) as well as canned eel (e.g. in Portugal) and for consumption as glass eel (e.g. in Spain). For the differentiation of the four eel species in fresh, smoked and canned products, Rehbein et al. [76] developed PCR-based techniques by means of universal primers for a fragment of the cytochrome *b* gene, RFLP and SSCP analysis of fragments.

Gadoids is the common name used for a group of bony fish, included in the taxonomic order of Gadiformes, which comprises several families of great commercial interest. The family Gadidae includes the Atlantic cod (*Gadus morhua*), the pollack (*Pollachius pollachius*) and the haddock (*Melanogrammus aeglefinus*). Calo-Mata et al. [69] developed a PCR-RFLP technique for the differentiation of 15 gadoid species, with the exception of *Gadus ogac* and *G. macrocephalus*, which could not be differentiated. These authors proved that the RFLP patterns obtained with three restriction enzymes can be used in the identification of commercial samples, namely salted fish labelled as cod. The amplification of a short fragment of cytochrom *b* gene and analysis by RFLP, SSCP and denaturant gradient gel electrophoresis allowed the differentiation of eight species commercialised as cod-fish [19]. Di Finizio et al. [86] developed a PCR-RFLP technique targeting the 16*S* RNA gene, which was able to identify seven gadoid species under study. Using sequencing and PCR-RFLP analysis of mitochondrial DNA control region sequences, it was possible to discriminate eleven hake species [73]. The quantification of haddock in raw fish or lightly cooked products was achieved by real-time PCR, whose calculated values obtained for model samples were within 7% of the actual value [87].

Tuna species include fish belonging to the *Thunnus* genus as well as to the genera *Sarda*, *Katsuwonus* and *Euthynnus*, whose species can have different market prices, depending on the country. When the morphological characteristics are removed by filleting or processing, such as canning, the only possibility to authenticate the food product is by the use of a molecular marker. The heat treatment by sterilisation, which canned products are subjected, results in the denaturation of muscle proteins becoming difficult or impossible the use of protein-based methods for species identification [67, 88]. The heat treatment of canned fish degrades DNA molecules to fragments between 100 and 200 bp [88, 89]. The analysis of amplified sequences from a region of the cytochrom *b* gene by sequencing and RFLP allowed the differentiation of tuna (*Thunnus albacares*, *T. alabunga* and *Katsuwonus pelamis*), bonito (*Euthynnus affinis*) and frigate mackerel (*Auxin thazard*) species in canned products [89]. In another work, the identification of six canned tuna species was achieved by using the same techniques [88]. Through a collaborative study, Rehbein et al. [90] showed that the analysis of a small amplicon from the cytochrome *b* gene by SSCP is a reliable technique for species identification in canned tuna. The novel TaqMan real-time PCR technique developed by Lopez and Pardo [91] allowed the quantitative detection of *T. alalunga* (albacore) and *T. albacares* (yellowfin), the two most valuable tuna species.

Species identification of sardine and other fish species used in canned sardine or sardine type products is important for the detection of commercial frauds. Jérôme et al. [92], through phylogenetic analyses of DNA sequences of *Sardine pilchardus* and some related species of genus *Clupeomorpha* in region of cytochrome *b* gene, developed a PCR-RFLP technique able to differentiate *S. pilchardus* from the other tested species, in fresh and canned products.

Sturgeon caviar is one of the most exclusive and expensive fishery products. Since the availability of the three most important and demanded species—beluga caviar (*Huso huso*), sevruga caviar (*Acipenser stellatus*) and osietra caviar (*Acipenser gueldenstaedti*)—has decreased over the last years, mislabelling of other less costly species became frequent. Therefore, reliable methods for species

identification are needed for the detection of frauds. The differentiation of ten sturgeon species in caviar of *Acipenser* and *Huso* was achieved by the development of a PCR-RFLP technique targeting the mitochondrial cytochrome *b* gene [93].

## Molluscs

The identification of commercialised mollusc species is also important to ensure the correct labelling of this type of food products. *Ruditapes decussatus* (grooved carpet shell), *Venerupis pullastra* (pullet carpet shell) and *Ruditapes philippinarum* (Japonese carpet shell) are among the most common clam species found in the market. *R. decussatus* is the clam species preferred by the consumers and also the most expensive, making it prone to fraudulent substitution, especially if the shell is removed and the clams are sold and mixed with other ingredients (usually frozen). For the differentiation of the three clam species, Fernández et al. [94] developed a PCR-RFLP technique targeting the α-actin gene. In another work developed by the same authors [95], the development of a PCR-SSCP targeting the same gene allowed the differentiation of grooved carpet shell and pullet carpet shell species.

Mussels are some of the most harvested and commercialised species of bivalve molluscs. They are widespread throughout the coasts of mild climate zones of the northern and southern hemispheres [96]. Mussels are a heterogeneous group that includes a high number of genera and each of them a great number of species. These genera are *Mytillus*, the most common species in prepared food products, *Perna*, *Aulacomya*, *Semimytilus*, *Perumytilus*¸ *Choromytilus* and *Brachidontes* [97]. As these bivalves are mostly commercialised frozen or canned without shells, the morphological identification becomes impossible, being subject to fraudulent labelling. A combination of PCR-based techniques, namely, PCR-RFLP, PCR sequencing and multiplex PCR was developed for mussel species identification in food products [96]. The differentiation of the above mentioned genera and the identification of five species (*M. californianus*, *Perna viridis*, *A. ater*, *Perumitylus purpuratus* and *S. algosus*) were achieved by amplification of nuclear genes 18*S* rDNA, forensically informative nucleotide sequencing (FINS) and RFLP analysis. The identification of four species of *Mytilus* (*M. edulis*, *M. trossulus*, *M. galloprovencialis* and *M. chilensis*) was succeeded by species-specific amplification of adhesive protein gene and RFLP analysis to discriminate *M. galloprovencialis* and *M. chilensis* species. The identification of the species *Perna canaliculus*, *P. perna*, *C. meridionalis* and *C. chorus*, was achieved by multiplex PCR targeting the ITS1 gene and RFLP analysis. The developed methodologies were validated with model products and applied to commercial samples [97]. RAPD analysis was applied to the identification of mussel species *M. edulis*, *M. chilensis*, *M. galloprovincialis* and *P. canaliculus*, which allowed designing species-specific primers and the differentiation of *M. galloprovincialis* from the other mussel species under study [97].

Cephalopods are part of the Mollusca phylum, from which squids are very popular seafood sold mainly eviscerated, skinned and cut into rings. The substitution of the most expensive species (*Loligo* sp) by species of the family *Ommastrephidae* is impossible to detect visually. Even within the family *Ommastrephidae* there are many species of different quality and price that could be employed for manufacture of squid rings [98]. Thus, the identification of commercial species is a relevant issue to assure the correct labelling of products. The identification of eight cephalopod species of families *Loliginidae* and *Ommastrephidae* was performed by two molecular techniques: FINS and PCR-RFLP [98]. By the use of FINS, it was possible to identify all the species in 17 commercial products.

## Dairy products

The authenticity assessment of dairy products is an important issue regarding the consumer's interests due not only to the economic point of view, but also to medical requirements, food allergies or religious practices. Common adulterations of dairy products are the substitution of higher value milk by nondeclared cow's milk or the omission of a declared milk species. Thus, the detection of milk species is important in cheese making, especially those made from one pure species and with protected designation of origin (PDO), such as pure sheep or pure goat's cheeses. In addition, some cheeses are manufactured with defined amounts of each type of milk [99–102].

Since Lipkin et al. [103] verified that somatic cells can be used as a source of DNA, several PCR-based methods have been developed for authenticity assessment of dairy products, allowing the differentiation of milks from different biological sources (cow, goat, sheep and buffalo) [104–107]. As amplifiable DNA can be obtained from thermally treated milks such as, pasteurised milk, ultra-pasteurised and powder milks, as well as cow's milk caseinates [18, 102, 106–108], cheese adulterations by partial or total substitution of non-declared milk species can be easily detected by PCR techniques. The resumed applications of PCR-based methods to dairy products authenticity are presented in Table 3.

Plath et al. [107] developed PCR-based techniques to differentiate cow, buffalo, goat and sheep β-casein genes. The results evidenced a higher sensitivity of PCR-RFLP technique than PCR-SSCP, allowing the detection of 0.5% (w/w) of cow's milk in ewe's and goat's milk cheeses.

**Table 3** Summary of PCR-based methods applied in the authentication of dairy products

| Food product | Species | Technique | Target gene | Limit of detection (%)[a] | References |
|---|---|---|---|---|---|
| Milks, mixture and pure species cheeses | Cow, sheep, goat, buffalo | PCR-RFLP | *β*-casein | 0.5 | [107] |
| | Cow, sheep, goat | Multiplex PCR/ PCR-RFLP | 12*S* rRNA/16*S* rRNA | 0.5 | [101] |
| Meat, milks and milk derivative | Cow, sheep, goat, buffalo | PCR-RFLP | Cytochrome *b* | NR | [121] |
| Mozzarella cheese | Cow, buffalo | Duplex PCR | Cytochrome *b* | 1 | [100] |
| | | Duplex PCR/PCR-RFLP | Cytochrome *b* | 1.5 | [111, 112] |
| | | Species-specific PCR | Cytochrome oxidise I | 0.5 | [113] |
| | | Species-specific PCR | 12*S* rRNA | 0.1 | [114] |
| | | Real-time PCR | Cytochrome *b*/growth hormone | 0.1 | [115] |
| Goat's and mixture cheeses | Cow | Species-specific PCR | *D-loop* | 0.1 | [99] |
| | Cow, goat | Duplex PCR | 12*S* rRNA | 0.1 | [119] |
| Sheep's and mixture cheeses | Cow, sheep | Duplex PCR | 12*S* rRNA/16*S* rRNA | 0.1 | [108] |
| | Goat, sheep | Species-specific PCR | 12*S* rRNA | 1 | [117] |
| Raw and heat treated milks | Cow | Species-specific PCR | 12*S* rRNA | 0.1 | [110] |
| | Goat, sheep | Species-specific PCR | 12*S* rRNA | 0.1 | [116] |
| | Goat, sheep | Real-time PCR | 12*S* rRNA | 0.5 | [118] |
| | Cow, sheep | Real-time PCR | 12*S* rRNA | 0.5 | [120] |
| Camember and Feta cheeses | Cow | Species-specific PCR | Cytochrome oxidase II/*D-loop*/ cytochrom *b*/12*S* rRNA | 0.5 | [106] |

*NR* not reported

[a] Values in percentage are expressed on a weight by weight basis

Other attempts have proved to be more successful when targeting sequences of mitochondrial genes. For adulteration detection of goat's cheese with cow's milk, Maudet and Taberlet [99] developed a species-specific PCR technique targeting a sequence from the control region of mitochondrial DNA, which allowed the detection of 0.1% (w/w) of cow's milk in model mixture cheeses. Another work developed by the same authors [109] reported the specific PCR detection of Prim'Holstein's milk, a cow's breed not allowed for cheese making of some French cheeses. The design of species-specific primers targeting the mitochondrial gene 12*S* rRNA enabled the specific detection of cow's milk in sheep and goat's milk mixtures [99]. The multiplex PCR technique developed by Bottero et al. [101] enabled the simultaneous detection of cow, goat and sheep's milk in cheeses inferred by the mitochondrial genes 12*S* and 16*S* rRNA. The application of the referred technique to commercial cheese samples evidenced the absence of one declared milk species (sheep) in three from 19 samples, confirmed by RFLP analysis.

The typical Italian Mozzarella cheese labelled with PDO should be produced only from pure water buffalo milk (*Bubalus bubalis*). As the addition of undeclared cow's milk to Mozzarella cheese is a common fraud, several PCR-based methods have been developed for that purpose. Two duplex PCR techniques targeting the cytochrome *b* gene were proposed for the simultaneous detection of cow and buffalo's milk in Mozzarella cheeses [100, 111]. The referred technique allowed the detection of 1% (w/w) of cow's in buffalo milk cheeses [100] and the application in the detection of commercial frauds with confirmation by RFLP analysis [111]. By a species-specific PCR targeting the cytochrome *b* it was detected the presence of undeclared cow's milk in 22 from 30 Mozzarella cheese samples with a sensitivity of 1.5% (w/w) [112]. Feligini et al. [113] and López-Calleja et al. [114] further improved the sensitivity of species-specific PCR assays for cow's milk detection in water buffalo milk cheeses to 0.5 and 0.1% (w/w), respectively. The validation and application of a real-time PCR technique for quantitative detection of cow's milk showed that most commercial Mozzarella cheese samples tested were contaminated with bovine milk [115].

The detection of undeclared goat's milk to avoid the substitution of ovine milk by lower cost milk such as goat's, was also achieved by PCR-based techniques. The species-specific PCR technique targeting the mitochondrial 12*S* rRNA gene [116] was successfully applied in the adulteration detection of ovine cheeses with goat's milk with a sensitivity of 1% (w/w) [117]. By using a real-time PCR technique, the same authors were able to quantify goat's milk in binary milk mixtures of goat/sheep in a range of 0.6–10% (w/w) [118].

The development of duplex PCR techniques for the quantitative detection of cow's milk in sheep [108] and goat's cheeses [119] allowed the detection of 0.1% (w/w) of cow's milk in those cheeses. By relating the fluorescent intensity of the target band (cow's) with a second band (sheep or goat), it was possible to normalise band intensities, because both products obtained by duplex PCR were equally affected by PCR inhibitors [108, 119]. Thus, reference normalised curves were obtained by plotting the relative fluorescent intensities versus the percentage of cow's milk of model cheeses in a range of 1–50% (w/w) for sheep's cheeses [108] and 1–60% (w/w) for goat's cheeses [119]. The developed techniques detected the absence of declared sheep and goat's milk in mixture cheeses. From nine commercial cheeses labelled with pure goat's milk, it was detected the addition of 9–13% (w/w) of cow's milk in three samples [108]. López-Calleja et al. [120] developed an approach by real-time PCR using TaqMan probes for the quantitative detection of cow's milk in binary milk mixtures of cow/sheep in a range of 0.5–10% (w/w).

Lanzilao et al. [121] developed a PCR-RFLP method targeting the cytochrome *b* gene for the identification of the four species (cow, sheep, goat and buffalo) of main interest in the dairy industry. The comparative analysis of 92 sequences from the cytochrome *b* gene allowed designing primers for a common fragment of the four species (275 bp). The RFLP patterns obtained with three restriction enzymes were distinct for each species.

Mayer [106] reported that species-specific PCR reveal to be a very sensitive technique for the detection of cow's milk, even in overripe mixed cheeses. Moreover, the PCR proved to be an adequate technique for the detection of heated dairy products, such as bovine milk and caseinate used to adulterate cheeses, conversely to protein based methods.

## Foods of plant origin

The PCR-based methods have important applications in species identification of foods, such as legumes [122–124], cereals [125] and allergens, with emphasis to nuts [126]. The applications include also cultivar identification in olive oil [127–129] and grape variety identification in musts [130–132]. Other particular applications of PCR-based methods in food authentication include the additives, such as the spices [133] and thickeners agents, such as locust bean gum [134]. The resumed applications of PCR-based methods to authenticity assessment of foods of plant origin are presented in Table 4.

Probably, the greatest number of applications in food analysis concerns GMO detection, mainly in soybean and maize derived food products, which are also emphasised in the present work.

### Olive oil

The olive oil is an increasingly important product due to its nutritional and commercial value, which is obtained from the olive tree drupes (*Olea europaea* L.) of several mixed or isolated cultivars. The olive oil typicity is obtained by the conjunction of climate conditions of a region and cultivar(s). Thus, some olive oils are protected by EU appellations PDO and protected geographic indication (PGI) according to the EU legislation [135].

Several works reporting the authenticity assessment of olive oil have been carried out for a long time, such as the analyses of sterols and fatty acids for elucidating adulterations of olive oils with seed oils [136, 137]. Analytical parameters independent from environmental fluctuations, such as DNA-based markers, have proved to be useful tools for the identification of the cultivar(s) used in olive oil production. The DNA isolated from processed foodstuffs, such as olive oil, can be highly degraded or rich in PCR inhibitors to be effectively analysed. However, several results evidenced acceptable levels of DNA amplification recovered from olive oil [127, 129, 138]. Busconi et al. [129] demonstrated that the DNA from olive oil can be used for amplified fragment length polymorphism (AFLP) analysis and that the profile of DNA purified from a monovarietal oil is equivalent to the profile from the leaves of the same cultivar.

Breton et al. [127] showed that DNA is present in all olive oil tested samples and even in refined oil, but the quantity may depend on the oil processing technology and oil conservation conditions. The comparison of several DNA extraction protocols showed that the method with magnetic beads allowed the highest DNA yields, which was successfully amplified by microsatellite primers and considered the best protocol for routine use [127]. The utilisation of SSR markers was found to distinguish virgin olive oils from different cultivars [128, 139]. The same technique clarified the presence of one cultivar in one PDO olive oil [141].

Doveri et al. [140] verified that care should be taken when interpreting DNA profiles from olive oil. DNA extracted from maternal tissues (leaves and olive pulp) showed identical genetic profiles by means of SSR markers. However, those authors found additional alleles in embryos (stone), also found in the paste obtained by crushing whole fruits and from oil pressed from this material. These results demonstrate that the DNA profile from olive oil is likely to represent a composite profile of the maternal alleles juxtaposed with alleles contributed by various pollen donors. Conversely, Muzzalupo et al. [141] demonstrated the absence of contamination of seed embryo DNA in monovariety olive oil.

**Table 4** Summary of PCR-based methods applied in the authentication of foods of plant origin

| Food product | Species | Technique | Target gene | Limit of detection[a] | References |
|---|---|---|---|---|---|
| Olive oil | *Olea europeae* cultivars | SSR/Real-time PCR | microsatellite DNA | NR | 127 |
| | | SSR | microsatellite DNA | NR | 128, 139, 140 |
| | | AFLP/RAPD | – | NR | 129 |
| | | RAPD | – | NR | 138 |
| | | SSR/Sequencing | microsatellite DNA | NR | 141 |
| Gluten in foods | Wheat | Species-specific PCR | NR | 0.1% | 142 |
| | Wheat, barley, rye | QC-PCR | chloroplast *trnL* intron | 0.2% | 144 |
| | Wheat, barley, rye, oats | Real-time PCR | $\omega$-gliadin (wheat), $\omega$-secalin (rye), hordey (barley), avenin (oat) | 50 pg DNA/0.01–0.1% | 145 |
| | Rye, triticale | Species-specific PCR/real-time PCR | $\omega$-secalin, chloroplast *trnL* | 8.28 pg DNA | 150 |
| Bread/pasta | Soft wheat, durum wheat | Duplex PCR | puroindoline b | 0.2% | 146 |
| | Durum wheat cultivares | SSR | microsatellite DNA | NR | 147 |
| | Soft wheat, durum wheat | SSR/species-specific PCR/real-time PCR | microsatellite DNA | 2.5% | 148 |
| | *Triticum* | Species-specific PCR/real-time PCR | gliadin, glutenin | 1% | 149 |
| Cereal foods and feeds | Wheat, rye, barley, oat, rice, maize (*Poaceae*) | PCR olinucleotide microarray | chloroplast *trnL* intron | 20–40 ng DNA | 151 |
| Food allergens | Hazelnut | Species-specific PCR | Cor a 1.0401 | 0.001% | 152 |
| | | PCR-ELISA | Cor a 1.0401 | $\leq$ 0.001% | 153 |
| | | PCR/PNA-HPLC | Cor a 1.0301 | 5 pg DNA | 154 |
| | | Real-time PCR | *hsp1* | 13 pg DNA/0.01% | 155 |
| | Peanut | Real-time PCR | Ara h 2 | < 0.001% | 156, 157 |
| | Hazelnut, peanut | Duplex PCR/PNA array | Cor a 1.0301, Ara h 2 | 50 pg DNA | 158 |
| | Walnut | Real-time PCR | Jug r2 | 0.24 ng DNA/0.01% | 159 |
| | Celery | Real-time PCR | mannitol dehydrogenase | 0.0005–0.001%, 0.01–0.001% | 160, 162 |
| | | Species-specific PCR | mannitol dehydrogenase | $\leq$ 1.53 ng DNA/0.1% | 161 |
| | Mustard | Real-time PCR | 2S albumin | 0.005% | 162 |
| | Sesame | Real-time PCR | *sinA* | 0.5 pg DNA/0.005% | 162 |

*NR* not reported

[a] Values in percentage are expressed on a weight by weight basis

## Cereals

The cereals such as wheat, rye and barley contain storage proteins (gluten) able to damage the small-intestinal mucosa of patients with celiac disease [126, 142]. A gluten-free diet is essential for these patients, thus adequate labelling is very important to avoid the inadvertent ingestion of gluten containing products. According to the draft revised standard for gluten-free foods of Codex Alimentarius Commission [143], "gluten-free" foods are foodstuffs consisting of or made only from ingredients which do not contain any prolamins from wheat or all *Triticum* species such as spelt, kamut or durum wheat, rye, barley, oats not exceeding 0.002% (w/w), or foods consisting of ingredients from wheat, rye, barley oats, spelt with a gluten level not exceeding 0.02% (w/w) on a dry matter basis. The methods used for the detection of gluten in foods are mostly based on protein analysis [126]. As alternative to immunological assays, Köppel et al. [142] used a species-specific PCR technique able to detect contaminations bellow 0.1% (w/w), whereas ELISA was about ten times less sensitive. A quantitative competitive PCR system was applied to 15 commercially available products labelled as "gluten-free" yielding identical results comparing to ELISA, in most of the cases [144]. Real-time PCR methods, using melting curve analysis for product identification, were established for the specific discrimination of wheat, rye, barley and oats in food samples, obtaining a good correlation with protein assays [145].

Italian traditional pasta is manufactured using durum wheat (*Triticum durum* or *Triticum turgidum subsp. durum*)

semolina, excluding the use of soft wheat (*T. aestivum*) to prepare this traditional food. Italian legislation permits a contamination of durum wheat by soft wheat in a maximum content of 3% (w/w) [146]. However, the use of soft wheat is frequent in other European countries, which demands sensitive techniques to protect the traditional pasta. For that purpose, a duplex PCR technique able to simultaneously detect both wheat species was successfully applied on high-temperature dried pasta, with a detection limit of 0.2% (w/w) of *T. aestivum* in *T. durum* [146]. Pasqualone et al. [147], by the use a set of five DNA microsatellites, were able to differentiate 20 cultivars of durum wheat. The application of SSR analysis enabled the selection of sequences to detect soft wheat in semolina and breads by species-specific PCR with a detection limit of 3 and 5% (w/w), respectively [148]. Using real-time PCR analysis the same authors lowered the detection limit to 2.5% and were able to quantify the adulteration of soft wheat in semolina [148]. Other strategies by means of real-time PCR with SYBR Green I dye and TaqMan probes allowed the detection and quantification of *Triticum* species [149] and rye [150] in raw and processed foods.

The differentiation of six important cereal species from the *Poaceae* family was obtained by amplification of the chloroplast *trnL* intron sequence with universal primers followed by a cyclic labelling of oligonucleotides probes and subsequent hybridisation to an oligonucleotide microarray [151]. The assay allowed the identification of mixed species in processed foods. The combination of the sensitivity of a universal PCR with the specificity of the labelling reaction provided a powerful tool for food authentication.

## Allergens

The labelling of foodstuffs for the presence of allergens is the only effective way to avoid the allergen-containing foods. However, total avoidance is sometimes difficult for the allergic individual, since processed food products contain a large variety of ingredients including allergenic foods. Thus, reliable detection and quantification methods for food allergens are necessary to ensure compliance with the food labelling and to improve consumer protection. Most of the commercially available and published techniques for allergen detection are based on the determination of potential allergenic proteins by immunological assays [126]. Although proteins are the allergenic components, DNA may be considered a marker for the presence of allergens.

Hazelnut is a potential food allergen often used as a food ingredient in pastry, confectionary products and ice-cream, being also processed to oils. Undeclared hazelnut might be present as cross-contaminant in processed foods. The species-specific PCR amplification of a fragment of the major hazelnut allergen Cor a 1.0401 gene allowed the detection of 0.001% (w/w) of hazelnut in commercial food products [152]. Holzhauser et al. [153] demonstrated that both enzyme immunoassays and DNA-based techniques, such as PCR-ELISA are powerful tools for allergen monitoring, namely hazelnut in processed foods at levels below 0.001% (w/w). Furthermore, PCR-ELISA showed a high specificity to hazelnut, with some advantage over protein assays when detecting hazelnut in some processed foods due to the higher stability of DNA. Another PCR-based technique for the specific detection of an internal region of the major hazelnut allergen was coupled with protein nucleic acid (PNA) probes and combined with high performance liquid chromatography (HPLC) analysis [154]. The PNA probes are analogues of oligonucleotides in which the sugar-phosphate backbone has been replaced by a pseudopeptide chain of *N*-aminoethylglycine monomers. The application of the PCR-PNA technique in the detection of hazelnut in vegetable and complex food matrices was found to successfully detect 5 pg of hazelnut DNA [154]. The use of real-time PCR technique with TaqMan probes for the detection of hazelnut in foods has been recently attempted, with a practical detection limit of 0.01% (w/w) hazelnut in model pastry samples [155].

Among all other foods, peanut allergy has earned the greatest attention in the medical and food production communities because of the high frequency and severity of adverse reactions in sensitised individuals [126]. The development of real-time PCR assays with TaqMan probes allowed the specific detection of traces of peanut in a range of food matrices [156, 157]. The analysis of a range of commercialised food products allowed the detection of undeclared peanut in 13% of the samples from 0.0001 to 0.0074% (w/w) [157]. A duplex PCR for the simultaneous detection of hazelnut and peanut in raw and processed foods enabled the specific detection of 50 pg of DNA of the targeted products [158]. For a more reliable detection of the two target species, the PCR products were hybridised with specific PNA probes in an array device, which enabled their detection and confirmation via specific sequence recognition [158].

Walnuts belong to widely consumed nuts used in bakery production as basic components of fillings in several cakes, in a range of confectionary products like filled chocolates, in cereal muesli mixtures and in other food products. Because walnuts contain allergens, they were recently introduced in the EU legislation, which requires the labelling of walnuts and products thereof [1]. Only one recently published work reports a PCR-based technique for walnut detection [159]. A real-time PCR technique targeting the major allergen gene of walnut allowed the detection of 0.24 ng of DNA and 0.01% (w/w) walnut in model pastry samples. When applying the referred technique to commercial

foods two samples with undeclared walnut were found in a total of 13 tested samples.

Celery is a plant extensively used as an ingredient in the food industry, mainly in products such as dried seasoning, dehydrated bouillons, sauces, sausages and ready-made meals [160]. As celery contains several identified allergens [126], the inadvertent consumption of celery due to improper labelling constitutes a considerable risk for allergic persons. For the detection of celery, Dovičovičová et al. [161] proposed a species-specific PCR technique targeting the mannitol dehydrogenase gene. The PCR technique showed specificity to four celery varieties with a detection limit $\leq$ 1.53 ng of DNA and 0.1% (w/w) in meat pâtés. A real-time PCR technique targeting the same gene was validated and successfully tested in more than 50 samples of foods, with a detection limit in model sausages of 0.0005–0.001% (w/w) [160]. Another real-time PCR technique for the specific detection of celery in foods has been recently proposed by Mustorp et al. [162], together with other applications to mustard and sesame, also considered as potential allergens [1]. Those authors present quantitative and sensitive real-time PCR methods using TaqMan probes able to detect 0.01–0.001% (w/w) for celery and 0.005% (w/w) for mustard and sesame in foods.

## Genetically modified organisms

During the past decade, the development of biotechnology has revolutionised agriculture by the introduction of genetically modified organisms (GMO). Since GMO entered the food chain, a scientific and public debate concerning their safety and the need for labelling information arose especially in Europe. For this reason, the EU has dedicated special attention to costumer information by requiring the compulsory labelling for food products containing more than 0.9% authorised GMO [163, 164]. Thus, the demanding for analytical methods has increased, not only in countries with labelling requirements, but also in those that want to export to countries with restrictions.

## GMO detection

The main strategies for GMO detection rely on two types of molecules: the proteins and nucleic acids. The DNA-based techniques, namely the PCR, are the methods of choice due to the higher stability of DNA when compared to proteins, together with the high sensitivity and specificity of PCR techniques [8, 11, 165–168].

The screening and/or detection of GMO by PCR-based techniques involve firstly a species-specific PCR as a reference target [169]. For example, in the case of soybean the detection of lectin gene is used as an endogenous control for the detection of roundup ready (RR) soybean [170–174], while the detection of GM maize lines involves the detection of invertase [170, 175, 176] and zein [174, 177–179] genes as endogenous control.

A typical gene insert (gene construct) in a GMO is composed of at least three elements: the promoter element functioning as a start signal, the gene of interest and the terminator element functioning as a stop signal for regulation of gene expression [10, 180]. In addition, several other elements can be present in a gene construct.

PCR-based GMO tests can be categorised into four levels of specificity: the screening methods, the gene specific methods, the construct specific methods and the event specific methods [10, 180]. The screening methods are the least specific and relate to target DNA elements, such as promoters and terminators that are present in many different GMO. The gene specific methods target a part of DNA harbouring the active gene with the specific genetic modification. These methods can provide information about the traits of a present GMO, but as the same gene can be used in several independent transformation events, they can not be used to determine whether the GMO is authorised or not. Both screening and gene specific methods are based on detection of more or less naturally occurring DNA sequences, which significantly increases the risk of obtaining false positive results [10].

The third level of specificity is the construct specific methods, which target the junction between two DNA elements, such as the promoter and the functional gene. The highest specificity is obtained when the target DNA is the unique junction found at the integration locus between the insert DNA and the recipient genome [10, 180].

Regardless the amplified target for GMO detection, the identification of the fragments for qualitative analysis involves usually agarose gel electrophoresis analysis. The confirmation of the fragments may be further verified by digestion with restriction enzymes and determination of size fragments [169, 171, 181, 182].

Multiplex PCR methods based on simultaneous amplification of multiple sequences save considerable time and effort by decreasing the number of reactions required to assess the possible presence of GMO in a food sample. Several approaches based on multiplex PCR have been developed for the detection of genetic modified (GM) maize lines [170, 173, 174, 176, 183], GM canola lines [176] and RR soybean [165, 173, 174]. Hernández et al. [183] described a multiplex PCR technique able to detect several GM maize lines (Bt11, MON810, T25 e GA21), which proved to be 100% specific to the events Bt11, MON810 and GA21.

In the recent years, the development of DNA-based new technologies for GMO detection aim to improve the conventional qualitative PCR techniques, avoiding the time-consuming subsequent gel electrophoresis step. In order to

meet the challenge of the steadily increasing number of GMO, new DNA-based technologies are coupled with multiplex assays. The combination of multiplex PCR coupled with ligation detection reaction (LDR) and microarray technology have been successfully applied in the simultaneous detection of recombinant DNA sequences (RR soybean and MON810, Bt176, Bt11 and GA21 maize) and endogenous plant genes (soybean lectin and maize zein) [184–186]. Other applications using microarray technology have been developed via PCR and PNA hybridisation targeting single [187] or multiple GM events [188]. Leimanis et al. [189] reported a multiplex DNA microarray chip with colorimetric detection for the simultaneous identification of nine GMO, five plant species and three GMO screening elements. Applications of microarray devices and multiplex PCR coupled with oligonucleotide fluorescent probes have been reported for the simultaneous detection of several GM events for RR soybean [190, 192, 193] and for maize [191–193].

Biosensors, for their characteristics of fast response time and low cost, are very attractive for new application in different emergent fields like GMO detection [194]. The combination of PCR amplification and biosensors when applied to GMO detection have been based on optical (Surface Plasmon Resonance), electrochemical and piezoelectric devices. The conjugation of oligonucleotides with gold nanoparticles used as probes constitutes an integral part of a DNA biosensor for the visual, rapid and sensitive screening of GMO [195].

García-Cañas et al. [196] explored for the first time the benefits of multiplex PCR followed by capillary gel electrophoresis with laser-induced fluorescent (CGE-LIF) for the simultaneous detection of GM maize events (Bt11, T25, MON810, GA25 and Bt176). The method proved to be highly sensitive and reproducible, demonstrating that can solve the artefacts of gel electrophoresis analysis.

### GMO quantification

The first attempts for GMO quantification in foods were based on the QC-PCR technique. Applications to quantitative detection of RR soybean [197–199], Bt176 maize [197, 198], Bt11 maize [200, 201], 35S promoter and NOS terminator [202] have been developed using QC-PCR.

At present, real-time PCR is the most commonly used technology for GMO quantification. The advantages of real-time-PCR, namely the reduction of time and artefacts by elimination of agarose gel electrophoresis analysis and the increased specificity conferred by the use of probes, makes it the technique of choice for GMO quantification [10, 20, 203]. Validation of real-time PCR techniques are generally accomplished by analysing certified reference materials (IRMM—Institute for Reference Materials and Measurements, Geel, Belgium), which represent mixture of flours containing defined proportions of GMO derived material. The quantitative assays are then applied directly to processed foods. However, DNA degradation occurs as a result of food processing decreasing the recovery of target sequences [200]. Engel et al. [21] reported that experiments demonstrating the influence of food composition and processing on relative quantification of GMO are still lacking. The quantitative GMO analysis should be expressed as the percentage of GM-DNA copy numbers in relation to target taxon specific DNA copy number calculated in terms of haploid genomes, as recently stated by the European Network of GMO Laboratories (ENGL) [204].

Currently, several techniques based on real-time PCR have been developed for GMO quantification. The quantification of RR soybean [170, 172, 178, 203, 205–207], GM maize lines such as Bt176 [175, 178, 209], Bt11 [175, 209, 210], MON810 [175, 179, 209, 211–213], GA21 [209, 214], T25 [175, 209], NK603 [179, 209], MON863 [209, 215], TC-1507 [209, 216], CBH351 [209], GM rapeseed [217, 218] and GM cotton [219] have been reported. Among the available detection technologies, TaqMan probes are the most used [170, 172, 175, 178, 179, 205, 207–219]. Other chemistries such as SYBR Green I [208, 213, 214], Scorpion primers [205], FRET [206], Molecular Beacons [208, 213] and AmpliFluor [213, 214] have also been reported for GMO quantitative detection.

## Conclusion

The technological advances are continuously offering novel strategies for analysis and authenticity assessment of foods. The DNA-based methods, namely the PCR, proved to be reliable, fast, sensitive and extremely specific techniques for the detection of frauds. The answers to the increasing demands of consumers concerning the presence of allergens, GMO, bovine meat and adulterations can be certainly obtained by the PCR technique.

The use of emergent technologies such as microarrays or chip-based systems coupled with PCR amplification represents a high potential due to their easy, speed and specificity. Furthermore, the miniaturised and automated nature of microarray technology is suitable for a great number of targets, although the analysis of data may become a complex task [167]. Thus, these new technologies are promising tools for multiple species identification in complex matrices, answering to the constant needs for GMO detection associated to the increasing number of approvals. However, quantitative applications of microarray technologies are still limited.

The increasing demands imposed by EU legislation in relation to foods labelling require quantitative methods. For

this purpose, real-time quantitative PCR techniques offer reliable, sensitive and specific protocols, as alternative to protein-based methods currently used for species identification. The simultaneous detection and confirmation of amplified fragments is a major advantage when using specific probes or labelled primers. Another advantage of real-time PCR is associated with the amplification of small fragments, which is more adequate to highly processed food products, favouring the kinetics of reaction and reducing the time of analysis [14]. The availability of a great number of equipments for real-time PCR, including software for data analysis has also contributed for the preference of this technology.

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