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Review

Animal species identification in food products: Evolution of biomolecular methods

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Abstract

Species identification in food has increasingly acquired importance due to public health, economic and legal concerns. Traditional methods have relied on the identification of morphological traits, but this does not lead to accurate identification of those species used in many types of processed food. As a result, laboratory techniques have been devised using electrophoretic and immunological methods focussing on protein profiles and, more recently, biomolecular techniques have been developed. However, these techniques also present problems and difficulties, especially in the case of matrices that are heterogeneous or have been subjected to severe treatments during processing.

Keywords: Animal species identification; Food; Biomolecular techniques

Introduction

Species identification in food for human and animal consumption has gained increasing interest in recent years due to various public health crises, such as the occurrence of bovine spongiform encephalopathy (BSE), avian influenza, swine influenza and dioxin contamination. These problems created panic among consumers, resulting in drastic reductions in consumption of beef, pork and poultry (Ciampolini et al., 2000 and Goffaux et al., 2005). The high incidence of food-borne disease due to microbial contamination of processed food has also produced food scares (Opara and Mazaud, 2001). Furthermore, the new food habits of younger generations and the progressive decline of organoleptic meat properties are important reasons for a negative trend in the consumption of food of animal origin (Cozzi and Ragno, 2003).

Conversely, there has been a recent consumer trend towards an enhanced interest in high quality products, resulting in a thriving trade in Protected Designation of Origin (PDO) products, meat from valuable species or breeds, 'high quality' milk, 'organic' products or food manufactured following traditional or local practices. Consumption of game meat has gained increasing popularity among consumers, who appreciate its texture and flavour, as well as its low fat and cholesterol content and the lack of anabolic steroids or other drugs (La Neve et al., 2008).

Topical issues include (1) food fraud related to substitution of one species by a similar but cheaper one; (2) protection of endangered species, and (3) assurance to consumers about their choices according to lifestyle (vegetarian), religion (absence of pork for Jews and Muslims) or health (absence of milk proteins, lactose or gluten for individuals with particular insensitivities or allergies).

For these reasons, great attention is now paid to the labelling of food, especially processed food, for which the discrimination of different ingredients is difficult (Woolfe and Primrose, 2004). For example, species identification in fish and fish products is essential at the time of their commercialisation. European regulation 104/2000/EC1 establishes that fish products can enter the food chain only if the commercial name, method of production and capture area are clearly labelled. Generally, identification of fish products is based on morphological characteristics, but fish can exhibit substantial morphological plasticity, even within the same species. Furthermore, identification of fish species by morphological characteristics has become more difficult due to globalisation of the food trade, which has introduced unknown species into European markets.

Morphological examination may be adequate to identify fresh fish, but it fails in the case of fish fillets, semi-processed products and canned products, which currently represent a high proportion of seafood marketing (Bottero et al., 2007). As a result, reliable diagnostic procedures are required to identify and/or authenticate food items of animal origin, thus protecting animals, producers and consumers (Pascal and Mahe, 2001).

Since the 1980s, many molecular and immunological techniques for the identification of species in food products have been developed. Initially, many tests were directed towards the identification of protein fractions in foods, including isoelectric focussing and ELISAs (Ansfield et al., 2000,

Mackie et al., 2000, Hurley et al., 2004, Mayer, 2005, Berrini et al., 2006 and Asensio et al., 2008). However, these techniques have been generally abandoned because of their low specificity and unsuitability in the case of complex matrices subjected to processing, such as chilling, salting, seasoning and, most importantly, heat, which induces marked structural modification of proteins.

Application of biomolecular techniques

These difficulties have stimulated application to the food domain of biomolecular techniques already successfully applied in other fields, such as forensic medicine. In the last decade, numerous protocols for DNA analysis for species identification in food matrices have been developed. Biomolecular techniques are now widely applied for the control of food for human and animal consumption, but such techniques can also present problems and difficulties, especially in the case of heterogeneous matrices or foods subjected to severe treatments during processing.

DNA extraction can be critical when the sample is not representative, in which case the DNA target may be present in very small quantities compared to the total amount of DNA extracted. Issues to take into account in these situations are (1) choice of target gene, (2) highly efficient methods of DNA extraction, and (3) highly sensitive detection methods. In addition, certain foods may contain substances inhibiting PCR (e.g. glycogen, fat milk proteins, collagen, polysaccharides and products of the Maillard reaction). This makes it necessary to apply (1) extraction methods able to remove these inhibiting substances, (2) additional methods of purification, and (3) addition of substances, such as bovine serum albumin, to PCR reagents (Hänni et al., 1995). These additional measures can affect the time and cost of analysis.

The heterogeneity of various types of food (e.g. meat, eggs) and the variety of certain products (e.g. the great number of different cheeses) have not favoured the development of specific commercial kits, since novel substrates often require the development of new extraction techniques or the adaptation of existing techniques.

During processing, food products might be subjected to thermal treatments (e.g. cooking, pasteurisation and sterilisation), high pressure, pH modification, irradiation and drying. Identification methods using such highly degraded substrate should be based on the analysis of very short DNA fragments, preferably 100–200 base pairs (bp). However, some analyses, such as sequencing, require amplification of longer fragments to increase the reliability of the result. Unseld et al. (1995) extracted DNA from canned tuna muscle and amplified a short mitochondrial *cytochrome b* (cyt b) sequence of 59 bp. The sequences obtained from this short fragment could not be used to differentiate all tuna species; in particular, two species of substantial commercial importance, yellowfin tuna (Thunnus albacares) and Atlantic bluefin tuna (Thunnus thynnus), were indistinguishable.

Selection of DNA marker

The choice of DNA marker is extremely important. The mitochondrial genome has been targetted preferentially for molecular diagnostics because it offers many advantages compared to nuclear DNA. In particular, the presence of mitochondrial DNA in multiple copies in cells increases the likelihood of detecting a specific sequence, even when it is present in small amounts, and also increases the chance of amplifying undamaged fragments of DNA, even after denaturing treatments.

Mitochondrial DNA genes (e.g. *cyt b*) have been widely used in phylogenetic studies (Kocher et al., 1989), confirming their value as markers in species identification, increasing the number of reference sequences in data banks and permitting better evaluation of inter- and intra-specific variability. Nevertheless, in the case of species of little interest from scientific, zootechnical or economic points of view, the number of sequences available for comparison is sometimes limited. Next generation sequencing techniques allowing rapid sequencing of whole genomes will improve the amount of sequence information available in data banks.

The nuclear gene for 5S ribosomal RNA (5S rDNA) was initially employed as a marker for the genetic identification of related species because it converts into a species specific structure in the higher eukaryotes (Rodriguez et al., 2001). The 5S rDNA gene gives origin to a multigene family of tandem arrays, whose unit of repetition is composed of one conserved coding region of 120 bp and a non-transcribed spacer (NTS) region that is variable in length and sequence among different species (Suzuki et al., 1996). Species-specific amplification by PCR can be achieved using primers located in the NTS. Cross et al. (2006) developed a multiplex PCR based on 5S rDNA gene sequences for identification of four oyster species (*Ostrea edulis*, *Ostrea stentina*, *Crassostrea angulata* and *Crassostrea gigas*).

The length of the NTS can sometimes be species-specific. Using a PCR with universal primers designed from the conserved coding region of the NTS region, Dalmasso et al. (2006) was able to differentiate skipjack tuna (*Euthynnus pelamis*) from *Thunnus* spp.

Polymerase chain reaction

The choice of the technique to be applied is based on the results of sequence alignments. Specific PCR seems to be best as a 'routine' test, because it is easy, rapid and allows the discrimination of several species at the same time (multiplex PCR), as well as the analysis of mixed matrices. It is considered to be a robust method, mainly in comparison with other methods based on the analysis of single nucleotide polymorphisms (SNPs).

Specific primers have been successfully applied to detect target species in a single or multiplex format. Some years ago, 'animal universal primers' designed from consensus sequences of the mitochondrial 16S rRNA gene were widely employed in specific PCRs to detect meals of animal origin in feedstuffs banned in Europe after the BSE crisis (European regulation 248/2002/EC2; Bottero et al., 2003b). This test has the advantage over conventional microscopic methods in that it can detect blood in feedstuffs, whereas microscopic methods were based on the presence of bone fragments.

PCRs using 'animal universal primers' have a drawback in that the presence of milk powder in feedstuffs, authorised by law, could hide the fraudulent addition of other meals of animal origin. In these cases, it is necessary to associate the biomolecular test with the conventional assay. Furthermore, DNA from the person performing the analysis could act as a target for the 'animal universal primers', leading to contamination of PCR products with human sequences.

In 2003, there was a change in the BSE regulations such that some animal meals can now be used in the manufacture of feedstuffs (European regulation 1234/2003/EC3). A multiplex test able to discriminate permitted products (meals from pigs, fish and avian species) from banned products

(meals of ruminant origin) has been developed (Dalmasso et al., 2004). This test is able to identify zoological class and can also distinguish ruminant DNA from other mammalian DNA.

Multiplex PCR can be applied advantageously to dairy products, since generally only a few species are used for milk production (cow, goat, sheep and buffalo) and it is unlikely that milk from other species would be present. Simultaneous identification of bovine, caprine and ovine DNA in a single assay has been applied to milk and dairy products (Bottero et al., 2003a).

Application of duplex PCR for species identification in 'mozzarella di bufala campana' has been described recently (Bottero et al., 2002). This product, which has obtained the PDO mark, is a high-grade cheese typical of southern Italy, in which water buffalo (*Bubalus bubalus*) milk is used exclusively (European regulation 1107/96/EC 4).

The main difficulty in the development of a multiplex PCR is the difference in length of the amplified fragments; they should differ in length by 40–50 bp to permit adequate resolution of fragments by agarose gel electrophoresis. Other difficulties can arise when samples are subjected to severe heat treatments. In these cases, DNA fragmentation reduces the number of species that can be identified at the same time to four or five.

PCR–restriction fragment length polymorphisms

Another strategy that can be used when species to be discriminated are closely related is analysis of SNPs. A widely applied technique is PCR–restriction fragment length polymorphism (PCR–RFLP), in which DNA amplified by PCR is digested with restriction endonucleases (Pereira et al., 2008). PCR–RFLP analysis of a conserved sequence of the mitochondrial 12S rRNA gene has been used to identify several closely related domestic and game species (Wolf et al., 1999, Pfeiffer et al., 2004 and Fajardo et al., 2006).

A combination of several enzymes is often required to discriminate between closely related species or when several species have to be differentiated simultaneously, precluding automation of PCR–RFLP (Ram et al., 1996, Quinteiro et al., 1998, Wolf et al., 1999 and Pardo and Pérez-Villareal, 2004). Furthermore, secondary conformation can prevent digestion of PCR products by restriction endonucleases (Quinteiro et al., 1998). Artefacts can occur when total DNA is analysed by PCR–RFLP due to amplification of non-translated pseudogenes that result from transfer of the mitochondrial DNA gene cyt b into the nuclear genome (Meyer et al., 1995 and Bottero et al., 2003a). These pseudogenes accumulate mutations, which can alter the distribution of restriction sites. This co-amplification phenomenon has been described in deer (Partis et al., 2000).

Mini-sequencing

Bottero et al. (2007) developed a mini-sequencing method to differentiate closely related species of tuna in canned products. This assay is based on dideoxy nucleotide triphosphate single base extension of an unlabelled sequencing primer at the 3′ end of the base immediately adjacent to a diagnostic site in a previously amplified fragment. This technique is as precise and reliable as sequencing and can be interpreted immediately. Discrimination of phylogenetically related species is possible with a single reading of the pattern generated by mini-sequencing. The potential for simultaneous interrogation of different diagnostic sites and automation make this assay a valid alternative to PCR–RFLP, especially when several species are to be identified. Reale et al. (2008)

described a mini-sequencing assay for species-specific detection of DNA from cattle, sheep, goats and water buffalo in milk and dairy products.

Allelic discrimination test

Mini-sequencing is not readily applicable to mixed matrices, mainly when species DNA is represented in different percentages. Dalmasso et al. (2010) developed a fluorescent qualitative test based on SNP analysis using a real-time PCR allelic discrimination TaqMan minor groove binding (MGB) assay. This method enabled identification of bovine and buffalo DNA in pure buffalo cheese products using a single assay. The advantage of this technique is that it does not require post-PCR manipulation. In addition, it permits analysis of mixed matrices, even though in this case only two species can be analysed simultaneously.

Sequencing

When the matrix is completely unknown and organoleptic characteristics are not fully ascribable to the putative species of origin, the diagnosis of 'presence' or 'absence' of a species is not sufficient. In this case, sequencing using universal primers is the logical option. The sequence derived by this approach is then compared with GenBank sequences to determine if there is a species match. However, the validity of the result can be compromised when the consensus sequence is not present in the data base, as may be the case with rare species.

In addition, sequencing is not applicable to mixed matrices unless different PCR products can be cloned separately and therefore this approach is not suitable for routine diagnostic testing. Sequencing of the cytochrome oxidase subunit I gene by forensically informative nucleotide sequencing has been used to identify all species in the fish genus *Lophius* (Espiñeira et al., 2008), several Australian fish species (Ward et al., 2005), 20 species of sharks and seven species of rays (Holmes et al., 2009).

Quantitative DNA analysis

New developments in quantitative analysis based on real-time PCR offer the potential for rapid results, high throughput, high sensitivity and opportunities for automation (López-Calleja et al., 2007a, López-Calleja et al., 2007b, Zhang et al., 2007, Kesmen et al., 2009 and Mininni et al., 2009). Most qualitative techniques have used mitochondrial DNA as the target, since it meets the requirement of high sensitivity. However, there are problems with the use of mitochondrial genes as molecular markers for quantification of species-specific DNA due to the variable number of copies of mitochondrial genes per cell among different species, different individuals of the same species and different tissues of the same individual (Lopparelli et al., 2007). As a consequence, it would be inadvisable to use mitochondrial DNA as the target for quantitative analysis of species-specific DNA in animal meals derived from whole carcasses. To overcome this problem, Walker et al. (2003) employed the 'short interspersed element' (SINE) present on nuclear DNA as a target for the quantification of bovine, porcine, chicken and ruminant DNA in feedstuffs.

In other food matrices, the problem of mitochondrial DNA variability can be overcome. Lopparelli et al. (2007) developed a real-time PCR method able to quantify the addition of bovine milk to pure buffalo cheese products, such as PDO buffalo mozzarella, using a normalised procedure based on two targets, namely, (1) the mitochondrial *cyt b* gene and (2) the nuclear growth hormone (GH)

gene; the latter target was selected because it is a single copy gene that is conserved between cattle and buffalo.

Variations in somatic cell counts (SSC) can lead to difficulties in quantifying DNA in milk. The concentration of somatic cells in milk depends on a number of factors, including age, stage of lactation, stress, season and udder health, particularly mastitis. Due to variability of SCC in individual samples, a linear correlation between the amount of added milk and the amount of detected DNA cannot be made. However, since milk and other dairy products are processed from 'bulk' milk, errors in quantifying DNA can be mitigated.

Sensitivity of biomolecular techniques

Specificity and sensitivity are important considerations in the development of diagnostic tests for detection of species-specific DNA. However, in the area of food control, high sensitivity can represent a problem in certain circumstances. As an example, the presence of bovine DNA in a 'pure buffalo' mozzarella cheese manufactured under strict control was attributed to cross-contamination, since the milk of both species was processed in the same plant (Bottero et al., 2002). In these cases, it is difficult to distinguish inadvertent cross contamination from fraudulent handling. A complete separation of the lines of production when food items of different species are processed is desirable, particularly in the case of PDO products.

Conclusions

Novel biomolecular approaches have opened up new possibilities for determining the species composition of food products. The choice of the most appropriate method for species identification is based on consideration of sensitivity, specificity, accuracy, discriminatory ability, reproducibility, speed, cost, availability of equipment and availability of suitably trained staff.

Conflict of interest statement

Neither of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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