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Food forensics: using DNA technology to combat misdescription and fraud

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The fraudulent misdescription of food contents on product labels is a widespread problem, particularly with high added-value products commanding a premium price. Proving conclusively that fraud has occurred requires the detection and quantification of food constituents. These are often biochemically similar to the materials they replace, making their identification and measurement extremely difficult. Despite the fact that food matrices are extremely complex and variable, a variety of the molecular markers used to physically map genomes have now been successfully adapted for detection of food substitution. These successes include the speciation of meats, fish and fruit in processed food products, the identification of the geographical origin of olive oil, the detection of dilution of Basmati rice with non-Basmati varieties and the quantitative detection of neuronal tissue and offal in processed meat.

Consumers require clear and accurate information to make informed choices about their diet and the foods they buy. The information given to consumers is essential for them choosing one food product over another. Consumer choice might also reflect lifestyle or religious concerns (e.g. vegetarianism, preference for organic products, absence of pork for Jews and Muslims), or health concerns (e.g. absence of peanuts, lactose or gluten for individuals with particular allergies). Therefore, the description and/or labelling of food must be honest and accurate, particularly if the food has been processed removing the ability to distinguish one ingredient from another. The information that must be given is enshrined in law in most developed countries, so that food supplied must be exactly what the labelling says it is. That is, the food must be authentic and not misdescribed.

There are several ways in which food can be misdescribed: (i) substitution of one ingredient by a similar but cheaper one (Food Standards Agency Food Surveillance Information Sheet Number 11/93; http://archive.food.gov.uk/maff/archive/food/infsheet/1993/no11/11fish.htm); (ii) extending or adulteration of food with a base ingredient, such as water; (iii) the non-declaration of processes, for example previous freezing or irradiation; and (iv) over-declaring a quantitative ingredient declaration. Some examples of substitution of high quality materials with ones of lower value are given in Box 1. Given that premium produce can command a significant price differential compared with the corresponding substituting ingredient, it is easy to see the financial gains that can be made by unscrupulous food producers [1,2].

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Box 1. Examples of premium foodstuffs

Basmati rice

Basmati rice is prized for its distinctive aroma and taste, its long thin grains and its distinctive cooking qualities. The varieties used to produce Basmati rice originate in the Himalayan foothills and have been subjected to centuries of cultivation and selection. Although these varieties produce a gastronomically superior grain, they have several undesirable agronomic traits: (i) lack of response to fertilizer, (ii) photoperiod sensitivity and (iii) difficulty in harvesting because of plant height and a weak stem. To counter these defects, Basmati varieties have been crossed with modern, improved varieties of long grain rice to generate hybrid Basmati varieties. Both types of varieties are approved and considered as Basmati, but the true-line varieties attract a higher price. In addition there are some long grain varieties of rice that superficially look like Basmati, but do not have its characteristic properties [22]. Therefore it is necessary to be able to distinguish between the different varieties of Basmati and long grain rice.

Virgin olive oil

Virgin olive oils are obtained from the olive using only mechanical means that do not alter the oil in any way [23]. The oil has not undergone any treatment other than washing, decanting, centrifuging and filtering. The definition 'virgin' excludes oils obtained by the use of solvents, deodorization and other refining techniques. The definitions 'extra virgin' and 'virgin' are based on a European marketing standard that includes fatty acid content and organoleptic characteristics. The oil also can have a designation of origin when it meets the specific characteristics associated with a region. 'Refined olive oil' is an oil of lower quality obtained by refining methods that remove excess acidity. 'Olive oil' is a low cost blend consisting mostly of refined olive oil and a small amount of virgin olive oil.

Tuna

There are several different closely related members of the tuna family, including albacore, yellowfin and skipjack. These can be sold as canned products or as fresh fish. There are premium varieties of tuna, such as the northern bluefin and southern bluefin tunas, which are prized for their size, texture, colour, fat content and taste, and fetch high prices on the Japanese market. However, the majority of canned tuna is made up of the more common varieties such as yellowfin and skipjack. In the same Scomberaceae family are many other species of fish classed as 'bonito', which fetch a much lower price when canned. Substitution of tuna by bonito in canned product has been a problem in the past (Food Standards Agency Food Surveillance Information Sheet Number1/00 http://archive.food.gov.uk/fsainfsheet/2000/no1/1tuna.htm).

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Methods for detecting misdescription and fraud

Detecting the total substitution of one ingredient usually requires a simple yes or no answer to the question 'Is this foodstuff or ingredient what I believe it to be?' Investigating partial substitution or adulteration is considerably more difficult for two reasons. First, in many cases it is necessary to know the possible identity of the adulterant before it can be detected. Second, for enforcement purposes, and to decide whether it is adventitious mixing or deliberate substitution, the amount of adulterant present is usually confirmed.

Many different chemical and biochemical techniques have been developed for determining the authenticity of food and in recent years methods based on DNA analysis have become more important. This is because some techniques, such as immunoassays, work well with raw foods but lose their discrimination when applied to cooked or highly processed foods. Also many techniques do not easily distinguish between closely related materials at the chemical level. For example, olive and hazelnut oils are similar chemically so the usual analytical methods cannot be applied to detect the adulteration of olive oils with hazelnut oil. Conventional chemical methods are also not always able to detect country or region of origin of olive oil. DNA analysis has discriminating power because ultimately the definition of a variety or species is dependant on the sequence of the DNA in its genome. DNA is more resilient to destruction by food processing (particularly cooking and sterilization) than other marker substances.

Technical considerations

Currently all the DNA-based methods for determining the authenticity of food make use of PCR. There are several technical considerations specific to the use of PCR for amplifying DNA derived from food. First, in many instances the test samples will be highly processed and might have been heated to temperatures $>100°C$ to cook or sterilize them. This results in DNA degradation and so PCR primers should be designed to amplify fragments of 200 bp or less [3]. Second, across the spectrum of foodstuffs to be examined, many different food matrices will be encountered including those high in oils, fats, vegetable material, animal tissue and various additives and fillers. The test sample might contain only raw ingredients or some or all of the components might have been milled, boiled, dried and so on. This means that DNA extraction procedures have to be optimized before analysis begins to ensure that sufficient test DNA is extracted and that inhibitors of the PCR are reduced or eliminated. To date, no single extraction method has proved useful with all the different matrices encountered. This problem is made more acute because quantification of the amounts of DNA from each species present often is required (Box 2).

Determining the identity of foods

Methods in food analysis have taken advantage of the rapid development of DNA techniques, but only a few methods have proved robust enough to be used. There are two approaches using PCR, which have proved very useful. One is the detection of single nucleotide polymorphisms (SNPs) that give rise to restriction fragment length polymorphism (RFLPs). Because PCR is used to amplify the fragments before cleavage, the sequences are sometimes known as cleavable amplifiable polymorphic sequences (CAPS) [4]. The other is the detection of small sequence length polymorphisms (SSLPs), otherwise known as variable number tandem repeats (VNTRs). In both these methods the final products are visualized using gel electrophoresis. By using fluorescent primers SSLP analysis can be made quantitative under certain conditions, but most quantitative techniques rely on real-time PCR.

Box 2. Quantitative PCR

Although the PCR is dependent on the concentration of the target, end-point detection of PCR products might not give an accurate measurement of the amount of target found in the original sample. This is because the amount of PCR product does not increase in a linear fashion and a plateau is reached.

Competitive PCR [18,24] relies on the amplification of an internal standard at the same time as the target DNA. This standard acts as a competitor to the target sequence during the reaction and direct comparison of products generated from the two targets indicates the amount of target in the sample. Equivalence is reached when the amount of standard and target yield the same amount of product. If fluorescently labelled primers are used and the PCR is restricted to the linear part of the amplification reaction, then it is possible to obtain a quantitative result by analyzing the products in a capillary electrophoresis system or a capillary DNA sequencer. This approach has been used to determine how much of a non-Basmati variety of rice is mixed with a Basmati variety.

In real-time PCR [18,24], a fluorescent reporter molecule is included in the assay mix and this enables the products of the PCR reaction to be measured after each cycle once a threshold has been passed. This reporter molecule is an oligonucleotide that has a fluorescent dye (reporter) attached to the 5′ end and a fluorescence quencher attached to the 3′ end and is designed to anneal to a position between the two primers. During amplification, the 5′-3′ exonuclease activity of the Taq polymerase digests the probe and releases the reporter molecule that then fluoresces. The amount of fluorescence produced is proportional to the amount of amplicon produced during PCR. DNA quantification is based on the threshold cycle (Ct), which is the cycle at which the fluorescence is detected at a predetermined value above the background.

In practice, a Ct value is determined for both a household (normalizing) target sequence that is non-discriminatory (acts as an internal standard) as well as for the specific target. To improve precision, both assays should be performed simultaneously within the same tube by using different fluorescent reporter molecules. The best approach to quantification is to have reference materials in which the adulterant is present at different levels and to construct a calibration curve based on the difference in Ct values for the two separate assays.

The accuracy of quantification obtained with PCR is largely dependent on the reference material used to construct the standard curve. For example, certified reference material is available for GM soya. That is, different levels of GM soya in non-GM soya, and has been prepared from full fat soya flour. As such, it might be unsuitable for use as a standard for GM soya that has been processed.

Identifying meat species in processed food products

Meyer *et al.* [5] described the use of the CAPS technique for the detection of pork in cooked meat products. In this instance the RFLP detected was in the gene encoding cytochrome b. This method has been widely used for speciation testing of meat products. Later, Hunt *et al.* [6] used species-specific satellite DNA sequences as probes in

Southern blots and identified rabbit and goat meats and lamb, pork and beef in raw, cooked and canned products. Today, the use of the gene encoding cytochrome b is nearly universal for determining the species of animals and birds in meat products [7] and fish. However, in the case of meats, CAPS analysis has been replaced with the TaqMan® assay, which detects SNPs using fluorescently labelled probes. The gene encoding cytochrome b is located on mitochondrial DNA and thus has two advantages: first, it is present in multiple copies in every cell thus making its detection easier; second, the mitochondria are likely to remain intact during processing (at least of raw food) thereby minimizing DNA degradation [3].

Determining the identity of fresh fish and fish species in processed products

The identification of fish can be problematic when morphological characteristics, such as the head, skin and fins are removed, as they are before or during processing. A well-used method for identifying raw fish is the characterization of water-soluble proteins using electrophoresis, but this cannot be used when the fish has been heavily heat processed. Nor is it always useful in distinguishing closely related species, for example salmon and trout.

Several different DNA-based methods have been used for identifying fish species. For example, Rehbein *et al.* [8] used single-strand conformation polymorphism analysis of three amplicons of the gene encoding cytochrome b to differentiate between various species of tuna and bonitos processed as canned fish. This differentiation is important because certain species command premium prices (Box 1). Later, this group [9] simplified the method by sequencing a 464 bp amplicon of the gene encoding cytochrome b and selecting six RFLPs that could be used to differentiate 10 salmon species. Twelve European laboratories evaluated the method by attempting to identify 10 unknown samples by comparison with RFLP profiles of authentic species. The success rate in this study was 96% [10] and subsequently the method was used to correctly identify the species within mixed and processed fish samples. Later, this 464 bp amplicon was found to be highly polymorphic in flatfish and this enabled the correct identification of 21 out of 24 species [10]. The method has also been used to discriminate between closely related species of hake, eel and sardines.

Distinguishing between different varieties of Basmati rice and long grain rice

An initial investigation [11,12] showed that a portfolio of 12 SSLPs could be used to differentiate Basmati from other types of long grain rice but could not completely differentiate true-line and hybrid Basmati varieties. To improve the differentiation, a further 39 microsatellite markers were selected from chromosomal regions linked to the distinctive traits of Basmati rice. When three of these microsatellites were combined with the original 12 it was possible to easily identify each Basmati rice variety (http://www.food.gov.uk/multimedia/pdfs/riceanalysis.pdf). If fluorescently labelled primers for microsatellite SSLPs are used then a quantitative determination is possible (Box 2).

Identifying potato varieties

Potatoes are a staple part of many Western diets and are eaten after cooking in different ways: boiling, baking, roasting and frying. A large number of potato varieties are available commercially and many have specific end-uses (e.g. baking, processing) or are prized for their taste and texture. Under current EU legislation there is a requirement for potatoes that are offered for wholesale or retail sale to be labelled with their variety name. This is both for consumer protection and a form of quality control and hence there is a need for a reliable method of varietal identification. The traditional method for identifying potato varieties has been analysis of the morphological characteristics of the tubers and/or the growing plants. However, this does not have the robustness necessary for enforcement purposes.

Electrophoretic protein and isozyme profiles have been used for identifying potato varieties but the method lacks robustness because the profiles are affected by the growth and storage conditions of the tubers. The analysis of DNA should obviate these problems and initially methods based on RFLP analysis and RAPDs were developed [13–15]. However, RFLP analysis is a lengthy procedure involving Southern blotting with labelled probes and RAPDs are not robust and reproducible. When these techniques were replaced with SSLP analysis, Corbett *et al.* [16] were able to differentiate 50 commonly grown UK potato varieties representing a diverse spread in origin and time. This method has been developed into a standard protocol.

The provenance of olive oil

Olive oils labelled with their region of origin sell at a price premium. This premium is greatest for oil from those regions associated with superior taste, consistency or colour. For cold-pressed oils (extra virgin and virgin) these properties are associated with the cultivar of olive tree and the soil (locality) in which it grows. In reality, only the cultivar is of relevance because only certain cultivars are grown in each locality. Therefore, if it is possible to determine the olive cultivar or cultivars used in the production of the oil then its geographical origin should be established.

Sefc *et al.* [17] described the use of microsatellites in characterizing olive cultivars from various regions of Spain and Italy. Using this approach, they distinguished six out of seven olive cultivars from other regions of Italy. Using PCR and the primers for these microsatellites, olive DNA was isolated from cold-pressed oils. The microsatellite profiles obtained with the oil-derived DNA generally were consistent with the cultivar used to produce the oil although some ambiguities were observed, for which there are two possible explanations. First, there could be variation within given olive varieties and this was detected because the trees sampled were not necessarily the ones used to make the oil. Alternatively, different cultivars might share the same name in different regions. Although this was a limited study, it suggests that it should be possible to use microsatellites to determine the provenance of high quality olive oils.

The need for quantitative PCR

The need to measure how much of a substituted ingredient is present is essential either for checking whether adventitious mixing has occurred or determining whether a limit set by law or custom has been surpassed. It can also be used for checking the amounts of different species that are present or checking a quantitative declaration. For example labelling is required if an ingredient contains >0.9% of an authorized genetically modified (GM) component, and new labelling requirements mean that meat products will declare their meat content in the form: pork (50%), beef (10%).

Durum wheat pasta substituted with common wheat

An example of the quantitative approach is the analysis of pasta and semolina. These foodstuffs should be prepared only with flour derived from *Triticum durum* (hard wheat). Because flour from *T. durum* sells at a premium, producers have been known to adulterate it with cheaper flour from common wheat (*T. aestivum*). This adulterated flour produces inferior pasta. Established methods based on high-performance liquid chromatography of proteins (gliadins) suffered from dependence on knowing the temperature of the drying regime given to the pasta. However, a method based on DNA analysis provides a more robust quantitative approach.

Common wheat is hexaploid and has three genomes (A, B and D) whereas *T. durum* is tetraploid and lacks the D genome [25]. A sequence (PSR 128) has been identified that shows a significant level of sequence polymorphism between the three genomes and little polymorphism within the genomes. In particular, there are 54 bp within an intron that is present in the D genome but not the A and B genomes. Thus the length of this intron can be used for detection of the D genome and a conserved region of the PSR 128 sequence can be used as an internal standard. For quantification, a set of reference pastas was prepared with hard flour adulterated to different degrees with soft flour.

Quantitative meat species determination

As noted earlier, sequences derived from the gene encoding cytochrome b are widely used to determine the animal species from which meat is derived. Because this gene is located on the mitochondrial genome it exists in multiple copies per cell and therefore is not suitable for quantitative studies. An alternative target for species identification is the single-copy gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A short sequence (<125 bp) from this gene can differentiate pork, beef, lamb, chicken and turkey. Assays based on the GAPDH gene can detect as little as 0.5% of one of these target species in the presence of any of the other species (http://www.chem.agilent.com/scripts/LiteraturePDF.asp?iWHID = 25742). The assays were also capable of detecting as little 5% of a target species in a canned meat mixture. The GAPDH gene assay has been used to quantify a particular species within sausages containing more meat from another species plus non-meat ingredients, such as rusk and potato starch. However, the assay consistently underestimated the amount of the 'contaminating' species and the most likely explanation is that the reference materials had a different composition. Nevertheless, the method can be used for enforcement purposes because it gives a minimum value for the contaminant with the true level being higher.

An alternative approach: quantifying fruit species by pyrosequencing

Fruit pulps are an essential ingredient of jams, flavoured yoghurts, fruit pies and desserts. Because the final product is highly processed it has been possible to hide the substitution of cheap pulps (e.g. blackberry or apple) for more expensive pulps (e.g. strawberry or raspberry). Now, polymorphisms in the chloroplast *rbcL* gene can be used for species identification. It is generally accepted that this gene is highly conserved. Various small regions of variation have been identified that permit small fragment amplification (as desired for processed products) and at the same time allow unique identification based on SNPs. When applied to jams and yoghurts it is possible to identify the various fruit species that are present. Furthermore, it is possible to make the test semi-quantitative by sequencing the amplified DNA using pyrosequencing [19–21]. Pyrosequencing is a method based on 'sequencing by synthesis' and the raw sequence data are presented as peaks with peak height reflecting the number of nucleotides incorporated. The close correlation between nucleotide incorporation and peak height can be used to determine how many of the template molecules have incorporated the added nucleotide, thereby allowing allele frequency determination.

Detecting neuronal and other non-muscle tissue in processed meat

The interest in detecting non-muscle tissue in processed meat products is driven by new European legislation that specifically excludes offal (liver, kidney and heart) from the definition of meat. Labels must declare the identity and content of non-muscle tissue and not conceal it within the heading 'meat'. The ability to detect neuronal tissue (from the brain and spinal cord) is also important because of labeling (to enable customer choice) and health (fears of new variants of CJD) concerns.

Detecting different tissues from the same species, and being able to quantify them, represents a unique challenge because all the tissues have exactly the same DNA sequence. The solution formulated by McDowell *et al.* was to exploit differences in the methylation status of gene promoters between muscle and non-muscle tissue. Such differences in promoter methylation, which occur at specific cytosine residues in a GC pair, are associated with the permanent and specific inactivation of genes, the activity of which is not required within these tissues. Differences in methylation patterns between DNA targets of the same sequence can be converted to different DNA sequences by chemical modification with bisulfite. The cytosine residues in DNA are converted to uracil by treatment with bisulfate, whereas methylcytosine residues remain unchanged. These differences in sequence following bisulfite treatment can be exploited by the use of specific primers in PCR-driven amplification.

The specific detection of neuronal tissue in the presence of homologous muscle tissue can be achieved by extracting

the DNA, treating it with bisulfite and then attempting to amplify DNA using primers specific for the site of methylation in the promoter for the gene encoding glial fibrillary acidic protein (GFAP). In tests with raw mixtures of tissues the system detected contamination of muscle tissue with as little as 0.01% of spinal cord tissue but was less sensitive for detecting brain tissue. In a similar manner, tissue derived from porcine liver and bovine liver, kidney and heart can be detected with primers for the promoter of the porcine connexin 32 gene. A more specific test for bovine heart tissue has been developed using primers for the promoter of the bovine gene encoding copper amine oxidase.

Summary

Robust DNA-based methods now exist for detecting or confirming the identity of various meat, poultry and fish species, for identifying potato varieties, for distinguishing true-line and hybrid Basmati rice varieties from other long grain rice and for detecting offal and neuronal tissue in processed meat products. These methods are being extended to the identification of premium tea varieties and the regional origin of cold-pressed olive oil.

Although methods that determine identity can be used to detect substitution, quantification of the level of substitution involves either the use of fluorescently labelled primers in the case of Basmati rice, or the use of real-time PCR. Currently it is possible to quantify GM soya in different food products, as well as common wheat in durum wheat pasta and different meat species in meat products.

Undoubtedly, the greatest challenges facing those who use DNA technology in food forensics are (i) the recovery of quality DNA from the vast array of complex food matrices and (ii) the impact of food processing on the size of DNA that can be recovered. The former will only be solved with the development of completely new DNA-isolation procedures rather than modifications to existing procedures. The solution to the latter lies in the development of suitable analytical procedures that do not involve DNA analysis. The rapidly developing discipline of metabolomics might provide such procedures.

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