

Detection of allergenic additives in processed meat products

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Abstract

Allergic responses to food components are an increasing problem all over the world. It is therefore important to protect people who are vulnerable to food allergens against accidental and unintended consumption of products containing allergic ingredients. The meat industry commonly uses various allergic additives in the production of processed products, such as legumes (soy, peas, beans), milk and egg preparations, cereals containing gluten (wheat, rye, barley and oats), and spices (celery and mustard). These meat additives have specific technological properties, which help to create a texture or flavor profile, or affect the nutritional value, although some of them, such as soy, mustard, milk and egg white proteins, can cause severe allergic reactions.

The aim of this paper is to discuss the application of various recently established methods of detection of allergenic additives in processed meat products – for instance cold cuts and sausages. The new methods are based mainly on protein, DNA, and isoflavones or phytic acid analysis. The article also characterizes the latest trends in the development of research on methods that would enable quick and reliable identification of targeted allergens in meat products.

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INTRODUCTION

Processed meat products are usually complex compositions, which contain not only meat, fat, and water but also a wide range of other ingredients. Usually these are salt, phosphates, vegetable proteins (soy, wheat flour), animal proteins (egg white, milk protein, blood plasma), hydrocolloids (gelatine, carrageenan, starch, dextrins, gums, pectins, cellulose), fiber, transglutaminase, and spices. Some of these substances are added purposefully due to their technological functions (condensation, gelation, emulsification, etc.), nutritional values or flavor. However, the common introduction of additives into the meat product is also aimed at reducing production costs.^{1,2} Additives may also be unintentionally placed in a product when the manufacturer fails to follow the procedures of good manufacturing practice (GMP) or good hygienic practice (GHP). Some of these additives belong to the group of products that might cause food allergic reactions.

A food allergy is an adverse immune response of an organism appearing each time that a specific allergenic ingredient is consumed. The main food components that usually elicit immunologic reaction are proteins originating from the 14 main food allergens.³ The mechanism of such reactions can be IgE-mediated, non-IgE mediated, or can combine both types. The symptoms of IgE-mediated allergic reactions develop immediately after exposure to the specific food and may cause gastrointestinal, respiratory, or dermatological reactions. In very severe cases anaphylactic shock may occur, even leading to death. The non-IgE mediated allergies are T-cell mediated often causing the inflammation of the gastrointestinal tract. Non-IgE allergy is very often not diagnosed

because the symptoms do not appear instantaneously after exposure to a given food product.⁴

The other type of undesirable reaction to food that can mimic food allergy is food intolerance. This kind of reaction does not exploit the immunological mechanism and usually gives a delayed response. Food intolerance can manifest some respiratory symptoms, rhinitis, urticarial, angioedema, gastrointestinal upset, headaches, migraine, fatigue, or musculoskeletal problems. Such reactions can arise due to carbohydrate (lactose, fructose, sorbitol) or food additive (methylparaoxybenzoate, sodium benzoate, sulfites) intolerance, among other reasons. In the case of vulnerable consumers, a food-intolerance reaction can be triggered by an intake of tyramine and vasoactive amines with cheeses or caffeine with beverages.^{4,5}

It is estimated that, at present, as much as 6% of the European population may suffer from food allergies.⁶

According to EU Regulation No. 1169/2011 of the European Parliament and Commission Directive 2007/68/EC, a package with food containing the additives that may cause different food allergies or intolerances should include the name of the food ingredients or other allergenic substances and this information should be legible to the consumer. Among 14 major protein substances

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or products causing allergies or intolerances included in Annex II of the EU Regulation mentioned above, seven major allergenic foods can commonly be added to meat products, namely soybeans, lupin, celery, mustard, cereals containing gluten, eggs and milk, and their products. As far as meat products are concerned, the manufacturer is obliged to provide information about the presence of proteins in a product and their origins, i.e. the animal species and type of protein (milk protein, collagen protein, etc.). In view of the growing market expectations to provide safe food to consumers, the development of a method verifying if the actual composition of a meat product is in agreement with the manufacturer's declarations is both a challenge and a necessity that science must face.

The aim of this article is to provide an overview of currently available analytical methods that can be used to identify common allergenic protein ingredients added to processed meat products. The article focused on the possibility of detecting selected additives that might cause severe allergic reactions to sensitive consumers, such as vegetable proteins (nuts, soy and gluten proteins), animal proteins (milk and egg proteins), spices (celery, mustard), and others.

ALLERGENIC ADDITIVES AND METHODS OF THEIR DETECTION IN PROCESSED MEAT PRODUCTS

Soy

The meat industry uses soy (*Glycine max*) preparations in the form of soy flour, soy concentrates and isolates, as well as textured soy. These additives gelate, emulsify, and stabilize the structure of the end product, reducing thermal drip and enhancing its water-holding capacity.⁷ From the nutritional point of view, they are a source of valuable protein. They can be also used as a fat substitutes by reducing the amount of calories in the final product.⁸ Soy is also rich in vitamins, especially B and E, and minerals such as calcium, magnesium, and iron.⁹ Despite its healthy properties, some countries have introduced limits on the maximum soy content in meat products. In Spain the amount of soy cannot exceed 30 g kg⁻¹ (dry weight).¹⁰ According to Portuguese law, frankfurter sausages cannot contain more than 50 g kg⁻¹ of vegetable proteins.¹¹

For a long time, scientists have been interested in the possibility of identifying non-meat and allergenic ingredients, such as soy and other legumes, in meat mixtures or meat products. The major soybean proteins responsible for allergenic reactions are seed-storage proteins Gly m Bd 30 K (formerly, 30 kDa Gly m 1), glycinin, beta-conglycinin, and also profilin, the Kunitz-trypsin inhibitor, Gly m 1, Gly m 2, and Bet v 1.¹²

Since the 1970s, electrophoresis has been used for such tests (Table 1). Hofmann and Penny¹³ tried to identify soy proteins with the one-dimensional SDS-PAGE method. As a result of electrophoretic separation conducted on polyacrylamide gels they obtained characteristic, noticeable bands of soy proteins with molecular weights of 53 and 17.5 kDa. The bands were visible and diverged from the animal proteins when the same analysis was carried out on mixtures containing beef, pork, and soy proteins, and they were also heated to a temperature of 100 °C. The molecular weight of the bands specific for pork and beef proteins differed from those derived from soy proteins. The bands received had a molecular weight of 40, 19 and 16 kDa. The bands were visible not so clearly when the samples were heated to a

temperature of 121 °C. Heat treatment affects, on the one hand, solubility of myofibrillar and sarcoplasmic proteins, which was ascribed to the increased hydrophobicity, but on the other hand it increases the degree of degradation of high-molecular-weight proteins. Protein-protein interactions caused by an increase in the protein surface hydrophobicity lead to their aggregation and coagulation, and damage to their tertiary and secondary structures, although the primary structure of proteins is often well preserved. As a result, the proteins became less prone to extraction in comparison with native proteins and the protein bands of heat treated samples are less visible on the gel after electrophoretic separation.²⁷ Lee *et al.*¹⁴ carried out a study to detect and measure the quantity of soy proteins mixed with meat whose content ranged from 50 to 350 g kg⁻¹ (both isolated and textured soy) in beef samples, heated at a temperature of 100 °C for 1 h.

Although one-dimensional electrophoretic methods enable the detection of additives in cold cuts, their use is currently limited. Small amounts of additives are normally used in the meat industry, and in these cases the limits of detection of electrophoretic methods are too high. Apart from that, allergenic agents may be found in insoluble mixtures of denatured proteins and other ingredients or they may be transformed during processing. Due to this fact, it may be difficult to interpret the result without ambiguity. For this reason, more modern methods gradually replaced one-dimensional electrophoresis, particularly for analysis of the composition of complex and thermally processed cold cuts and sausages.

At present, enzyme-linked immunosorbent assay (ELISA) is a method approved by the Association of Official Analytical Chemist (AOAC)²⁸ and recommended for the measurement of soy proteins in raw and heated meat products. However, due to protein denaturation in heated meat products, species identification using enzymatic methods can be sometimes a challenge. The processing operations (e.g. smoking, cooking, pasteurization, sterilization) can negatively affect the antigen-antibody reaction because of the susceptibility of the conformational epitopes to heating. The ELISA tests are thus relatively easy to perform and sensitive but there are some limitations in the case of heated samples.²⁷ This technique is based on the immune reaction so it enables the identification of a particular selected/targeted protein. The ELISA method enabled Macedo-Silva *et al.*¹⁵ to identify textured soy in beef hamburgers made for research purposes and soy in hamburgers purchased in chain stores, using antibodies recognizing two storage protein fractions, beta-conglycinin (7S), and glycinin (11S).

Immune methods are sensitive and their results are reliable particularly for products that are not heat treated. The problem could be the identification of proteins in thermally processed products. Due to the denaturation of proteins, they may be less soluble in extraction buffers than native proteins.²⁷ However, there are many commercially available kits testing for soy in all food products, raw materials, and processed foods based on immunochemical detection, such as sandwich ELISA or lateral flow tests.

In order to identify the content of soy in various foods, including meat products, antibodies specific to the soy protein Gly m Bd 30 K were used.¹⁶ The study focused on the problem of soy protein extraction from complex matrices of processed food products, especially thermally processed ones, where proteins lose their three-dimensional structure and their water solubility is reduced. The study proved that, to increase protein recovery, the extraction buffer composition could be improved by increasing the content of sodium dodecyl sulphate (SDS) to 20 g kg⁻¹. On the other hand, the indirect competitive ELISA test¹⁷ enabled the identification of

Table 1. Soy protein detection in meat products by application of immunological, genetic and separation methods

Foodstuff	Marker	Method	Limit of detection (g kg ⁻¹)	References
Soy protein, cooked meat mixtures	53 and 17.5 kDa molecular weight proteins	Electrophoresis	Not studied	Hofmann & Penny ¹³
Cooked meat mixtures	–	Electrophoresis	50–350	Lee et al. ¹⁴
Hamburgers	Beta-conglycinin (7S), glycinin (11S)	ELISA	Not studied	Macedo-Silva et al. ¹⁵
Meat products	Gly m Bd30K protein	ELISA	Not studied	Morishita et al. ¹⁶
Sausages	Soy protein	ELISA	5	Renčová & Tremlová ¹⁷
Meat products	Lectin gen	PCR	10	Meyer et al. ¹⁸
Raw meat products	Lectin gen	PCR	15	Soares et al. ¹⁹
cooked meat products				
Meat products (hamburgers, meatballs, nuggets, sausages)	Lectin gen	End-point PCR, real-time PCR	0.1–60	Soares et al. ¹¹
Meat products	Lectin gen	Tetraplex PCR	0.1	Safard et al. ²⁰
Meat products	Lectin gen	EvaGreen multiplex real-time PCR	0.01	Safard & Abasiyanik ²¹
Cooked meat products	Peak retention time: 1.7 min and 2 min	RP-HPLC	0.7	Castro-Rubio et al. ²²
Fermented sausages (<i>chorizo</i>)	Peak retention time: 1.7 min and 1.0 min	RP-HPLC	0.4–0.6	Criado et al. ²³
Cooked meat products	Peak retention time 1.7 min	RP-HPLC	4	Castro et al. ¹⁰
Cooked meat products	Isoflavones (genistein, daidzein)	RP-HPLC	1	Vranová ²⁴
Cooked meat products	Peak retention time 12.30 min	RP-HPLC	1.9	García et al. ²⁵
Poultry products (sausages, frankfurters, pâtés)	Peptides originating from glycinin, beta-conglycinin, sucrose-binding protein	Nano-LC-QTOF-MS/MS	Not studied	Montowska & Fornal ²⁶

5 g kg⁻¹ soy content in experimental meat products. The study also proved the possibility of identifying soy protein in commercially available products, such as different types of salami and other sausages.

Although ELISA is an approved method used for the identification of soy in meat products, there are also some other methods, such as chromatography, which can be applied for this purpose. Perfusion reversed-phase chromatography (RP-HPLC) and UV detection enabled the identification of two soy-specific peaks, whose retention times were 1.7 and 2 min. The method applied to identify soy proteins in model meat samples that were not heat processed resulted in content as low as 0.7 g kg⁻¹, whereas quantitative measurements revealed 0.25 g kg⁻¹ or higher content of soy. Soybean concentration was also determined in commercial thermally processed pork products. Employing the calibration curve, soy content was detected below 30 g kg⁻¹ in each analyzed sample, which is in agreement with Spanish regulations.²²

Previous studies showed that the detection of soy proteins based on the peak retention time of 1.70 min was possible, even in thermally processed products made from chicken and beef, and in mixtures of different meat species (chicken, pork, beef and turkey), and in the presence of milk proteins.¹⁰

The same method was applied to detect and measure the quantity of soy in cured meat products, such as pre-dried, fermented sausages (*chorizo*). Depending on the component of mobile phase used in tests, i.e. acetonitrile (ACN) or tetrahydrofuran (THF), the soy protein-specific peak retention time was 1.7 and 1.0 min, respectively. The detection limit was also very low and amounted to 0.4 g kg⁻¹ for ACN and 0.6 g kg⁻¹ for THF. Acetonitrile enabled measurement of the minimum soy quantity of 1.3 g kg⁻¹ whereas THF enabled measurement of the minimum quantity of 2.2 g kg⁻¹.²³ The differences result from the use of various components of the mobile phase in which the analyte is dissolved. Components used in mobile phases, such as ACN, methanol, isopropanol, or less commonly applied THF, differ in elution strength and also affect the efficiency of ionization of the analyte molecules, hence the difference in the results obtained in the above study when using ACN and THF.

The study conducted by García *et al.*²⁵ enabled the identification of 1.9 g kg⁻¹ soy content in thermally processed meat products by the RP-HPLC method and UV detection at 254 nm. The measurement of soy quantity with this method was possible when the minimum amount was 6.3 g kg⁻¹. The identification was based on

the peak retention time of about 12.30 min. The study simultaneously proved that the components of the matrix under analysis did not influence the results.

The usefulness of the RP-HPLC method with UV detector for the identification of soy content was also confirmed by Vranová.²⁴ The method applied in the study enabled the identification of 1 g kg⁻¹ soy content in thermally processed meat products, whereas quantitative identification was possible within the range of 1–100 g kg⁻¹. The study involved the analysis of isoflavones contained in soy, i.e. genistein and daidzein.

There are alternative methods based on DNA analysis and polymerase chain reaction (PCR). Above all, they are characterized by high sensitivity and specificity. Due to the higher thermal stability of DNA compared with protein epitopes in immunoassays these methods are often used for analysis of thermally processed food. Most studies on the application of genetic methods of soy protein detection refer to the lectin gene sequence. Initially, i.e. in the 1990s, the PCR technique identified 10 g kg⁻¹ soy content in meat products.¹⁸ Later it was possible to identify smaller amounts of the protein – less than 10 g kg⁻¹.

Studies based on the soy lectin gene sequence conducted by Soares *et al.*¹⁹ enabled the identification of 1 g kg⁻¹ soy protein content (hydrated textured soy) in unprocessed (unheated) pork samples and 5 g kg⁻¹ content in samples heated at 121 °C for 5 min. The same group of scientists continued their research by using end-point PCR and real-time PCR to examine meat products available in Portugal (hamburgers, meatballs, chicken nuggets, steamed and raw sausages) for the content of soy proteins ranging from 0.1 to 60 g kg⁻¹ (per dry soy protein).¹¹ The identification procedure confirmed that the results obtained for most of the samples were in agreement with Portuguese law. There were five samples (out of 42) where the content of soy protein exceeded permissible limits and one where measurements revealed soy content of 20 g kg⁻¹, although the producer declared no soy protein additive. Safdar²⁰ developed primers that can be used for detecting 0.1 g kg⁻¹ content of soy and three meat species (horsemeat, poultry, pork) in one PCR. As in the studies mentioned above, soy identification was based on the lectin gene sequence. Subsequently, the same group used EvaGreen multiplex real-time PCR,²¹ which is a method of quantitative DNA measurement, to detect 0.03 g kg⁻¹ beef content in model cold cuts and 0.01 g kg⁻¹ soy content during one PCR.

Cow's milk and egg proteins

The analysis of studies on the detection of additives in meat products shows that soy seems to be in the leading position. Nevertheless, there are also studies on the identification of other ingredients, such as animal proteins, i.e. milk and egg proteins. Milk proteins added to meat products improve their juiciness and texture and they subtly influence the flavor profile of a finished product.²⁹ Due to the gelling, emulsifying, and stabilizing properties of egg components they affect the consistency of the end product. Milk and egg proteins are also included into the group of products that are most often responsible for the occurrence of allergic reactions to consumers. The major allergenic cow's milk components are caseins and whey proteins, such as alpha-lactalbumin, beta-lactoglobulin, bovine serum albumin, immunoglobulin, and lactoferrin. In egg white, ovomucoid, ovalbumin, ovotransferrin, lysozyme, and in egg yolk alpha-livetin and YGP42 cause allergic reactions the most often.³⁰ Allergies due to these food ingredients are more common in children than in adults.^{5,31}

One of the first studies conducted in this area was based on electrophoretic methods, where electrophoresis was used not only to detect the soy content but also 5 g kg⁻¹ content of casein and egg white albumin in model beef products heated to 70 °C.³² It was impossible to identify this content of proteins in products heated to 120 °C due to the absence of clear, protein-specific peaks in the densitogram. Leduc *et al.*³³ used isoelectric focusing (IEF) combined with immunoblotting and the ELISA technique. The results showed that only ELISA successfully identified egg protein added to raw, pasteurized, and sterilized meat products. Immunoblotting preceded by IEF failed to detect this ingredient in sterilized products. Examples of detection of milk and egg proteins are presented in Table 2.

Recently, Petrášová *et al.*²⁹ applied immunofluorescence microscopy to detect milk proteins in heated meat products. The use of Texas Red fluorochrome resulted in fluorescence, which indicated a positive reaction between the antigen, i.e. beta-casein, and the biotin-marked antibody. The tests revealed milk proteins in 17 out of 20 samples where producers declared their presence. The researchers explained the absence of positive reaction in the other three samples by the fact that producers prematurely declared the presence of milk proteins in case the product was accidentally contaminated.

Due to the development of mass spectrometry techniques in the last decade, traditional electrophoretic techniques that were used for analyzing proteins have lost their importance. Combined techniques of high-performance liquid chromatography and mass spectrometry (LC-MS) are increasingly used to identify specific proteins of plant or animal origin in complex matrices of food products. Shotgun proteomics techniques have become particularly popular, where peptides obtained by digestion of complex protein mixtures are separated by means of liquid chromatography and then they are identified by means of tandem mass spectrometry (MS/MS). In recent years ambient mass spectrometry (AMS) techniques have also been in focus because they enable the identification of proteins and peptides without prior chromatographic separation. As a result, the time required for sample preparation and analysis may be considerably reduced. The authenticity of meat and processed meat products was analyzed by means of one of AMS techniques, i.e. liquid extraction surface analysis – mass spectrometry (LESA-MS).³⁴ This technique enabled the identification of numerous peptide markers characteristic of different meat species. Simultaneously, during the same analysis of products containing milk protein additives (cheese, milk preparations), which had been purchased from a supermarket, milk proteins and peptide markers specific to bovine casein alpha-S1 were detected and identified. Recently, a nano-LC-QTOF-MS/MS method was used to detect reliably allergic proteins derived from functional protein additives in various commercial poultry meat products, i.e. sausages, frankfurters, and pâtés.²⁶ Heat-stable peptide markers, unique to soy, milk, and egg white were detected. The peptides originated from main allergenic proteins, namely soy glycinin and beta-conglycinin, milk alpha-S1-casein and the whey protein beta-lactoglobulin, as well as egg white ovotransferrin and lysozyme C. These proteins appeared to be relatively resistant to meat processing including roasting and sterilization. These studies proved that while analyzing complex mixtures of proteins formed by digestion of processed meat products it was simultaneously possible to identify their species composition and to monitor other illegal practices related to adding non-meat and/or allergenic proteins.

Table 2. Detection of milk, egg and seafood proteins in foodstuff

Foodstuff	Marker	Allergenic ingredient	Methods	Limit of detection	References
Raw, pasteurized and sterilized meat products	Antigens and allergens in egg white	Chicken egg white	ELISA	0.3g kg ⁻¹ 0.3 g kg ⁻¹ 1.25 g kg ⁻¹	Leduc et al. ³³
Raw and pasteurized meat products			IEF/immuno-blotting	20 g kg ⁻¹	
Cooked meat products	Beta-casein	Milk proteins	Immunofluorescence method	Not studied	Petrášová et al. ²⁹
Meat products (sausages, frankfurters)	Casein alpha-S1	Milk proteins	Ambient mass spectrometry	Not studied	Montowska et al. ³⁴
Poultry products (sausages, frankfurters, pâtés)	Alpha-S1-casein, beta-lactoglobulin, ovotransferrin, lysozyme C	Milk and egg white	Nano-LC-QTOF-MS/MS	Not studied	Montowska & Fornal ²⁶
–	Beta-laktoglobulin	Milk proteins	SPR biosensor	Not studied	Wu et al. ³⁵
Wines	Ovalbumin	Chicken egg	SPR biosensor	30 µg L ⁻¹	Pilolli et al. ³⁶
Sardine fish cake, dried skipjack	Parvalbumin	Fish proteins	SPR biosensor	Not studied	Lu et al. ³⁷
Shrimps	Shrimp allergen	Shrimps proteins	Piezoelectric biosensor	333 µg L ⁻¹	Xiulan et al. ³⁸

The expectations and requirements of the modern world to simplify and accelerate all analyses led to the development of fast immunochemical assays, which can be conducted in a shorter time by less qualified laboratory staff. For example, the fast-strip test against egg white and ovalbumin developed by Baumgartner *et al.*³⁹ was supposed to enable the detection of egg proteins in food. The study proved that antibodies used for detecting proteins in the egg white were more sensitive than for detecting proteins contained in the yolk or the whole egg. Unfortunately, it was difficult to detect the presence of egg proteins in meat products. It may have been caused by the small amount of this ingredient in the product or its processing. It is still hard to obtain a simple and rapid method that does not compromise reliability and efficiency.

Celery and mustard

Celery (*Apium graveolens*) is added to meat and offal products or ready-to-eat meat dishes because of its unique taste. It contains substances that may cause acute allergic reactions such as Bet-v-1-homologous Api g 1, profilin (Api g 4), Api g 5.¹²

Mustard is another spice that is commonly added to meat products because it enhances flavor. In the case of white mustard (*Sinapis alba* L.), albumin Sin a 1 and globulin Sin a 2 are considered as allergens, whereas in brown mustard (*Brassica juncea*) protein Bra j 1 classified as albumin can cause allergic reactions.³⁰ Allergens in mustard are thermally stable and resistant to enzymes. It is estimated that up to 7% of food allergies may be caused by mustard. The occurrence of this allergy is particularly high in France, probably due to high mustard consumption.⁴⁰

To date, only a few studies concerning detection of celery as well as mustard in meat products have been reported, and they are summarized in Table 3.

The study conducted by Dovičovičová *et al.*⁴¹ is an example of research on the identification of celery in food products (meat pâtés). The researchers detected 1 g kg⁻¹ of celery content by real-time PCR and complementary primers to the mannitol dehydrogenase gene. Hupfer *et al.*⁴² used the same technique and the

same molecular target. They identified 0.005–0.01 g kg⁻¹ content of celery seeds in emulsion-type sausages.

Duplex real-time PCR enabled the detection of celery (*Apium graveolens*) and white mustard (*Sinapis alba* L.) mustard-seed protein in model sausages during one reaction. In raw sausages the technique enabled the detection of 0.05 g of celery and 0.01 g of mustard seeds kg⁻¹ of the product. In sausages heated to a temperature of 75–78 °C for 15 min the celery and mustard content of 0.05 g kg⁻¹ was detected.

The primers used in the reaction were based on mRNA for the MADS D protein for mustard seeds and on NADPH-dependent mannose-6-phosphate reductase for celery. They did not result in cross reactions with 64 different biological species, including those belonging to the parsley (*Apiaceae*) and mustard (*Brassicaceae*) families.⁴³

The identification of mustard is subject to research that mainly uses PCR methods. By using the primers and probe specific to the gene for reverse transcriptase from gypsy retroelement 13G42-26 it was possible to distinguish between the presence of black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*). It was possible to detect 0.05 g kg⁻¹ content of black mustard in model raw sausage and 0.01 g kg⁻¹ content in steamed sausage. As far as brown mustard is concerned, the content of 0.05 g kg⁻¹ was detected both in raw and heated cold cuts. The primers were not ideal because they caused a cross-reaction with white mustard (*Sinapis alba* L.). The primers also reacted to the DNA of such spices as cinnamon (*Cinnamomum zeylanicum*), cumin (*Cuminum cyminum*), fenugreek (*Trigonella foenum-graecum*), ginger (*Zingiber officinale*), turmeric (*Curcuma longa/domestica*), or rye (*Secale cereale*). However, as far as these spices are concerned, the occurrence of cross reactions does not affect the possibility to identify mustard. The difference in the threshold cycle value (Ct), i.e. the difference in the number of cycles that are necessary to generate the needed fluorescence to exceed the set threshold value for mustard and spices, was greater than 11.⁴⁴

Three varieties of mustard were also identified by means of duplex real-time PCR. As in the studies mentioned above, the detection of black and brown mustard was based on the gene

Table 3. Examples of celery, mustard, wheat and peanuts detection in foodstuff

Foodstuff	Marker	Allergenic ingredient	Methods	Limit of detection	References
Meat products (meat pâtés)	Mannitol dehydrogenase gene	Celery	Real-time PCR	1 g kg ⁻¹	Dovičovičová <i>et al.</i> ⁴¹
Meat products (emulsion-type sausage)	Mannitol dehydrogenase gene	Celery	Real-time PCR	0.005–0.01 g kg ⁻¹	Hupfer <i>et al.</i> ⁴²
Raw meat products	NADPH-dependent mannose-6-phosphate reductase	Celery	Duplex real-time PCR	0.05 g kg ⁻¹	Fuchs <i>et al.</i> ⁴³
Cooked meat products	mRNA for MADS D protein	Mustard		0.01 g kg ⁻¹	
Raw meat products				0.05 g kg ⁻¹	
Cooked meat products	Reverse transcriptase from gypsy retroelement 13G42-26 gene	Black mustard	Real-time PCR	0.05 g kg ⁻¹	Palle-Reisch <i>et al.</i> ⁴⁴
Raw meat products					
Cooked meat products		Brown mustard		0.01 g kg ⁻¹	
Raw meat products				0.05 g kg ⁻¹	
Cooked meat products	Reverse transcriptase from gypsy retroelement 13G42-26 gene	Black mustard	Duplex real-time PCR	0.001 g kg ⁻¹	Palle-Reisch <i>et al.</i> ⁴⁵
Cooked meat products					
Meat products	MADS D gene	Brown mustard	Real-time PCR	0.01 g kg ⁻¹	Zeltner <i>et al.</i> ⁴⁶
	Glutenin gene	White mustard		0.005 g kg ⁻¹	
Meat products	Isoflavones, phytic acid, galactooligosaccharides	Gluten	HPLC	Not studied	Vaňha <i>et al.</i> ⁹
Peanuts proteins	Ara h1	Peanuts	SPR biosensor	770 ng L ⁻¹	Wu <i>et al.</i> ³⁵
Chocolate candy bars	Ara h1	Peanuts	SPR biosensor	90 µg L ⁻¹	Pollet <i>et al.</i> ⁴⁷

sequence for reverse transcriptase from gypsy retroelement 13G42-26, whereas the detection of white mustard was based on the MADS D gene. The primers pair designed for black and brown mustard amplified 76 bp products size whereas in a case of white mustard 74 bp amplicon was generated.⁴⁵ In model steamed sausages containing black and white mustard the amounts of 0.001 and 0.01 g kg⁻¹ were detected, respectively.

Other additives

Cereals, i.e. flours made from cereals, are commonly used as additives in the meat industry. Wheat (*Triticum L.*) flour proteins are perfect binders and fillers of meat products because they contain gluten, which interacts with water and forms a spatial structure, which gives elasticity and ductility to the end product.^{48,49} Wheat proteins bind water very well and thus they naturally influence the juiciness and efficiency of the final product. However, the consumption of even a minimal amount of gluten may cause different clinical symptoms, e.g. hives, diarrhea, or even anaphylactic shock in people who do not tolerate this substance. People suffering from coeliac disease must definitely avoid food containing gluten.⁴⁶ According to EU Regulation No. 41/2009 of the European Parliament, gluten-free products must contain less than 0.02 g kg⁻¹ of gluten. Real-time PCR can be

used for detecting gluten, which enters into meat products using cereal-based preparations. This protein is identified by means of primers specific to the gene sequence of high molecular-weight glutenin. These primers are capable of detecting gluten in common wheat (*Triticum aestivum L.*), spelt (*Triticum spelta L.*), durum wheat (*Triticum durum Desf.*), and rye (*Secale cereale L.*). Studies proved that real-time PCR can detect the content of these cereals in meat filling when it is as low as 0.005 g kg⁻¹.⁴⁶ Examples of detection of the additives described in this section are included in Table 3.

Real-time PCR is also useful for detecting nuts in food. This group of products is commonly known for its allergenic effect but it is also a valuable ingredient due to its unique taste and nutritional values. The major allergens present in nuts are storage (vicilins, legumins and 2S albumins) and structural proteins (profilins, oleosins).³⁰ López-Calleja *et al.*⁵⁰ investigated 179 different samples of food, including meat products, for the content of hazelnuts (*Corylus avellana*). The identification was based on the ITS rDNA coding sequence. It enabled detection of the hazelnut content of less than 0.1 ppm.

The high-performance liquid chromatography (HPLC) technique was used for analyzing the content of isoflavones, phytic acid, and galactooligosaccharides. Their presence in meat products is

caused by the use of preparations of plant origin. Coarse soy flour, fine low fat soy flour, pea flour, chickpea flour, wheat flour, rice flour, commercial mixes for meat production, and meat products were investigated. The study proved the presence of isoflavones, i.e. genistein and daidzein, in preparations made from legumes. The study did not prove their presence in wheat flour or rice flour. As far as galactooligosaccharides are concerned, the greatest amount of raffinose (8.3 g kg^{-1}) was found in chickpea flour, stachyose – in soy products (38.7 g kg^{-1}) and verbascode – in pea flour (6.3 g kg^{-1}). Trace amounts of these compounds in rice flour and commercial mixes were ascertained. There were significant amounts of phytic acid in all of the preparations under analysis. The examination of meat products showed that greater amounts of these compounds in cheaper products were presented. The content of plant preparations was detected in 13 out of 18 meat products under analysis, but only seven producers declared their presence in the product composition.⁹

Biosensors – future prospects

Biosensors are devices that have a real chance to be used for the rapid detection of additives in meat products in near future. To date, biosensors have been used to detect pathogens, pesticides, the remains of drugs, heavy metals, and toxins in food.⁵¹ They are integrated systems composed of a receptor part and a transducer. The receptor part is the sensitive biological component, which may include enzymes, antibodies, and nucleic acids. The transducer constitutes the detector component, which converts biochemical signals into optical, electrochemical, acoustic or thermal signals, proportionally to the amount of the analyte under investigation.^{52,53} Regarding the analysis of allergic components in foods, several studies have been undertaken which showed the potential of biosensors for investigation of milk and peanuts,^{35,47} fish and shrimps,^{37,38} egg,³⁶ soy, and genetically modified soy^{54–56} (Tables 2 and 3).

Surface plasmon resonance (SPR) is a phenomenon that is often used in optical sensors for food analysis. This method measures reflected light intensity, which varies according to the number of molecules bound on the metal surface connecting centers of different optical density.⁵³ The SPR method was used to detect the ingredients that are responsible for the most common allergic reactions. This technique enabled the detection of milk and peanuts through the identification of protein of beta-lactoglobulins and Ara h1, Wu *et al.*,³⁵ and Pollet *et al.*,⁴⁷ respectively. It also identified the content of fish through the application of anti-parvalbumin murine monoclonal antibody (MAb) EG8, to detect the parvalbumin, which is the main allergen found in fish,³⁷ and the content of eggs through the detection of ovalbumins (OVA).³⁶

Electrochemical biosensors are also used for the detection of genetically modified organisms in food. A single-stranded DNA probe bound on the electrode surface is used in electrochemical sensors. The hybridization of the complementary DNA sequence in the test sample with the probe generates a measurable signal. Apart from that, the stage of DNA amplification by PCR can be skipped in this method because there is a sufficient amount of DNA extracted from the material under analysis. Such a simplification of the method considerably reduces the time required for analysis.⁵⁶ Biosensors using DNA hybridization allowed genetically modified and unmodified soy to be distinguished. Genetically modified soy was identified by means of a complementary probe to the sequence of 35S promoter and NOS terminator,

the sequences regulating the EPSPS gene expression entered into RR soy.⁵⁶

Acoustic sensors use piezoelectric quartz crystals. They induce acoustic resonance at a particular wave frequency in an alternating electric field. The resonance depends on the size and weight of the crystal. If the analyte is bound on the crystal surface, the resonance frequency changes.⁵⁷ These sensors enabled the identification of transgenic soy by means of probes specific to the P35S and NOS terminator sequence.⁵⁴ A probe binding to the EPSPS gene sequence itself can also be used for the identification of a transgenic soy variety.⁵⁵ Piezoelectric sensors also detected allergens found in shrimps.³⁸ The authors of the research stress the fact, that in contrast with conventional PCR, this method eliminates the need to use toxic reagents such as ethidium bromide. Apart from that, an adequately prepared sensor surface can be reused several times in further analyses.

Sensors with thermal transducers measure heat generated or absorbed in a biological reaction.⁵⁷ This method was used to detect sucrose, for example. The method of sucrose detection is based on the hydrolysis of this disaccharide with invertase, which is immobilized on the sensor surface.⁵⁸

Biosensors are an interesting alternative among food testing methods. They are comfortable to use because they can be operated easily. The device is small and there are minimal requirements concerning the preparation of a sample for tests. It is noteworthy that in a short time biosensors can provide reliable results for many simultaneously tested samples.⁵¹

SUMMARY

Consumers expect reliable information about food, including meat products. The meat industry uses a wide range of functional additives, which can positively affect the end-product quality if they are used in reasonable amounts. Consumers can benefit from the use of additives because they receive an attractive-looking, palatable product with a good texture; however, commonly used functional preparations are the source of major food allergens, i.e. legumes, milk, egg, celery proteins, and cereal gluten proteins. It is therefore important that customers should be clearly informed about the content of additives on each product label, and their use must be in agreement with the law. This is particularly important for people suffering from food allergies, who should feel safe to buy the products which producers declare to be free from allergenic ingredients. For this reason it is necessary to have analytical tools, which can verify the composition of food offered in the market.

In recent decades, there has been enormous progress in the development of methods verifying the composition of meat products. Some of them have lost their importance to new methods involving the application of genetic or mass-spectrometry-based techniques, or various types of biosensors, which offer greater efficiency and sensitivity of analysis. Researchers are attempting to develop a method that would give reliable results in a short time and with the least effort possible. The method would certainly be a perfect tool for monitoring food quality and it would guarantee that consumers have access to safe food.

At present there is still no perfect method that meets all of the requirements including high accuracy and sensitivity, low cost, and rapid analysis of complex and processed products. Electrophoretic techniques are time consuming and less sensitive. There are many commercially available ELISA tests for allergen detection in food. These enzymatic tests are relatively cheap

and sensitive but because they are based on immunological reactions their application for thermally treated products may sometimes give false-negative results. The methods employing DNA or mass spectrometry-based methods for protein analysis are accurate and sensitive but there are also some limitations such as time-consuming sample preparation, expensiveness and demand for well-trained and experienced staff. Perhaps in the future, in this particular area of research, biosensors will meet all the requirements mentioned above regarding accuracy, price, and convenience of handling.

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