SPECIES IDENTIFICATION OF MEAT AND MEAT PRODUCTS

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1. INTRODUCTION

Species identification is a recent analytical problem. Meat cuts of different species may not always be distinguished macroscopically from each other on basis of colour and texture only. For space and energy saving reasons, deboned and cut meat is more and more marketed, either frozen, boxed or vacuum packed. Frozen meat is often processed without previous thawing. This may encourage adulteration. In the meat industry and in the meat trade, there is a growing interest in simple and cheap methods for the control of this type of raw material. Ground meat may consist of a mixture of meat of different species. The determination of the relative proportion of meat of several species in a meat product represents a complicated analytical issue. The problem becomes even more complicated when the age (e.g. veal - beef), the sex (e.g. boar - sow) and / or the breed of the animal, from which the meat originates, is to be determined.

There are various reasons for which species identification is wanted or required. One of the most important is the difference in price between meat of various species, which can be significant. The use of MDM (mechanically deboned meat) in meat products is an example. Furthermore there are economical regulations which are different for meat of different species (e.g. in case of overproduction). In order to enforce such regulations, control is required. Species analysis may also be demanded for religious and ethical reasons. For instance, the consumption of pork or pork derived products is prohibited by islamic and jewish religious law. A certificate of complete absence of such products may be obligatory for exporting meat products to some of the countries adhering these religions. In the Anglo-Saxon countries, on the other hand, there is a dislike for horse meat.

The methods used for species identification can be divided into three groups. First there are the anatomical and histological methods. Based on his experience, a trained meat inspector must be able to recognize certain parts of muscles and meat cuts visually. Microscopic and electron-microscopic methods can also be used.

The immunological methods represent a second, highly important group used for species identification. These methods are based on the specificity of the antigen-antibody reaction. Until recently, only raw meat or meat products which were heated only moderately (< 60°) could be examined by these methods. Nowadays, techniques are improved allowing analysis of heated meat products too. A third group contains a large variety of physico-chemical methods. Meat proteins can be separated by electrophoresis or HPLC. The electrophoretogram or chromatogram shows a pattern of bands or peaks, some of which may be specific for one or more species. Species specific components or ratios of components can be analysed and determined quantitatively in most cases by appropriate chemometric methods. For the demonstration of the presence of a certain species, and for the quantification of the fraction of different species in a compound product, pattern recognition techniques are often used.

This paper reviews the different methods in use for species identification. Emphasis is put on the determination of species specific proteins and fats in meat products.

2. ANATOMICAL AND HISTOLOGICAL METHODS

Morphological examination can make a limited contribution to species identification. In general, this is only possible before processing of cuts of slaughter animals, edible offals etc. The typical question whether cuts are from a rabbit, or from a cat can be solved in this way. Similar questions may be asked for other species and answered by morphological examination.

Microscopical anatomy or histology offers some possibilities for the identification of organs, but is very restricted in species identification itself. Cutaneous mucous membranes may be recognized as such and may be

very valuable for qualitative evaluation. Determination of the species itself is very difficult and often impossible. Some organs and tissues show some species specific characteristics (e.g. red blood cells with oval nuclei in fowl). However, these cells are difficult to retrieve after processing (Lauwers, 1988). Within one species (e.g. chicken) histology may be used for the recognition of the origin of breast meat of chickens (broilers or spent hens) (Uijttenbogaart and Jansonius, 1986).

3. IMMUNOLOGICAL METHODS

Immunological analytical methods are based on the ability of higher animal species to develop antibodies (Ab) against foreign substances (antigens = Ag). These antibodies bind very specifically with the corresponding antigen. In this way, proteins, characteristic for a species can be demonstrated. The antibodies are raised by administration of the Ag to laboratory animals of another species (e.g. bovine albumin to rabbits), which will produce antibodies against the Ag. After several weeks the serum is collected and can then be used in an analytical test. A major advantage of immunological methods is their sensitivity and specificity. Meat mixtures of different species can be examined without previous separation of the proteins.

The two most appropriate immunological procedures for meat identification are the classical Ouchterlony double agar gel immuno-diffusion technique (AGID) and the enzyme linked immunosorbent assay (ELISA). The Ab-Ag binding is visualized by precipitation (AGID) or by an enzymatic colour-reaction (ELISA).

Double immunodiffusion (Ouchterlony, 1949; Shaw, 1983; Swart and Wilks, 1982) is a simple but convenient method for species identification. An aqueous extract of soluble proteins from an homogenized meat sample is prepared and placed in a well in an agarose gel. In a second well an antiserum, specific against meat of the species to be tested, is placed. During overnight incubation Ag and Ab diffuse. If both are present, a visible opaque band will appear in the gel between the wells at the position where Ab and Ag came in contact with each other.

In the ELISA method the Ag-Ab interaction occurs on the wall of a microwell and is made visible by the action of an enzyme chemically bound to one of the immunoreagents (Jones and Patterson, 1985 & 1986; Jones *et al.*, 1986; Whittaker *et al.*, 1983). Dilutions of meat extracts are added to plastic microwells, precoated with a preparation of purified species specific antibody. If proteins of the tested species are present in the meat extract, they will bind to the Ab attached to the well. The plate is then washed and a peroxidase conjugated second antibody is added. After incubation, excess conjugate is removed by washing and the bound peroxidase activity is determined by adding a fixed amount of substrate (a chromogene). Colour development is directly proportional to the original amount of specific protein in the extract.

4. PHYSICO-CHEMICAL METHODS

4.1. Electrophoretical techniques

The proteins from a meat extract can be separated by electrophoresis in a carrier gel (e.g. polyacrylamide gel : PAGE). A pattern of protein bands is obtained which can be visualized by staining. The separation of proteins by gel electrophoresis is based on their charge, size and molecular weight.

Iso-electric focusing (IEF) in polyacrylamide gel or in agarose gel is a more recent technique which finds many applications in species identification. Proteins are separated not on basis of differences in charge or mass, but on basis of differences in iso-electric points. In a gel, a pH gradient is established by means of ampholytes (mixtures of small molecules with a specific charge). The steepness of the pH-gradient has to be adjusted to the problem. With this method even closely related species may be clearly distinguished. Moreover, the composition of mixtures of meat from two or more species can be determined in some cases. In meat mixtures, the pre-

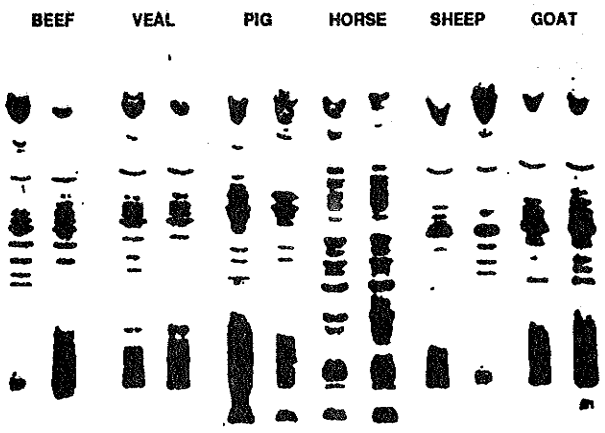

Fig. 1 Species identification by Agar Gel Iso-Electric Focusing

sence or absence of characteristic protein bands for the species to be determined, is observed. Fig. 1 gives an example of such a protein pattern. Several authors found that these electrophoretic methods are also very suitable for the differentiation of fish species (Abrams *et al.*, 1983, 1984).

Analysis of total sarcoplasmatic proteins can result in rather complicated patterns. The interpretation is sometimes difficult because the patterns will depend on the muscle type. Therefore, modifications of this technique were designed which produce a less complicated pattern. Hofmann (1986) described the separation of myoglobins from meat juice. After IEF of meat juice, most species show two main proteins bands and several minor bands. The position of the two red coloured myoglobin bands permits the visual identification of several species. Heat-treated meat products can also be analysed with this method (Bauer and Hofmann, 1987). An important advantage of this method is the simple way of sampling (meat juice) and the absence of time consuming (de)staining of the gel. Other techniques are based on the difference in iso-enzymes or on differences between the iso-electric point of certain enzymes in different species (adenylatekinase, creatinekinase) (King, 1984). Electrophoretic techniques are generally expensive and time consuming but they are valuable because specific antisera are not always available for all species.

4.2. High Performance Liquid Chromatography (HPLC)

Proteins of meat products can also be separated by HPLC (Ashoor and Osman, 1987). The meat product is usually extracted with twice its weight of distilled water. A part of the extract (20-25 µl) is injected into the liquid chromatograph. The separation is carried out on a reversed phase column with adapted solvents (Ashoor *et al.*, 1988; Osman *et al.*, 1987). The peaks can be detected with a UV-detector at 280 nm. The chromatogram consists of many peaks, some of which can be specific for a certain species. As is the case with electrophoresis, the different components (peaks) are generally not fully identified. They are characterized by their retention time which is usually expressed relative to that of BSA (Bovine Serum Albumin). The method is sensitive (e.g. detection of 1% pork meat in beef meat) and fast. However, the sample capacity is low. Only one sample / hour can be analysed. Another disadvantage is the variation of water soluble components within the different carcass sites.

4.3. Peptide analysis

In some meat products, the proteins are denatured by heat or other treatment. In that case, species identification by immunological methods, electrophoretical techniques or direct HPLC is difficult and sometimes impossible. Analysis must be carried out on heat stable components. The histidine containing peptides anserine (A) (ß-alanyl-1-methylhistidine), balenine (B) (ß-alanyl-3-methylhistidine) and carnosine (C) (ß-alanyl-L-histidine, sometimes called ophidine) are process stable and can be used for species identification.

Table I gives the A/C and B/A ratios of some important species. Pork, beef and horse meat show a low A/C ratio. In the chicken, kangaroo and rabbit, high A/C ratios are found. The anserine : carnosine (A/C) ratio can be used for the determination of chicken meat in cooked pork meat (up to 5%). Mixtures of pork and beef can also be analysed based on these ratios.

Adulteration of meat products with kangaroo meat (or chicken meat) can be proven by this method (Tinbergen and Slump, 1976).

The anserine : carnosine : balenine ratio (in fact the A/C and the B/A ratio) was used for the differentiation of pork, beef, horse, kangaroo and sheep meat (Carnegie *et al.*, 1982 & 1983). Within terrestrial animals, balenine is

Table I A/C and B/A ratios in different animals *

low A/C (B/A)		medium A/C (B/A)		high A/C (B/A)	
pig	0.02 - 0.1 (1.1)	sheep	0.6 - 0.9 (0.01)	chicken	2.2 - 5.5 (0)
cattle	0.06 - 0.2 (0.03)	goat	0.7 (0)	kangaroo	10 (0)
horse	0.01 (0)	lamb	1 (0.02)	rabbit	10 (0)

* (Tinbergen and Slump, 1976; Carnegie *et al.*, 1982 & 1983)

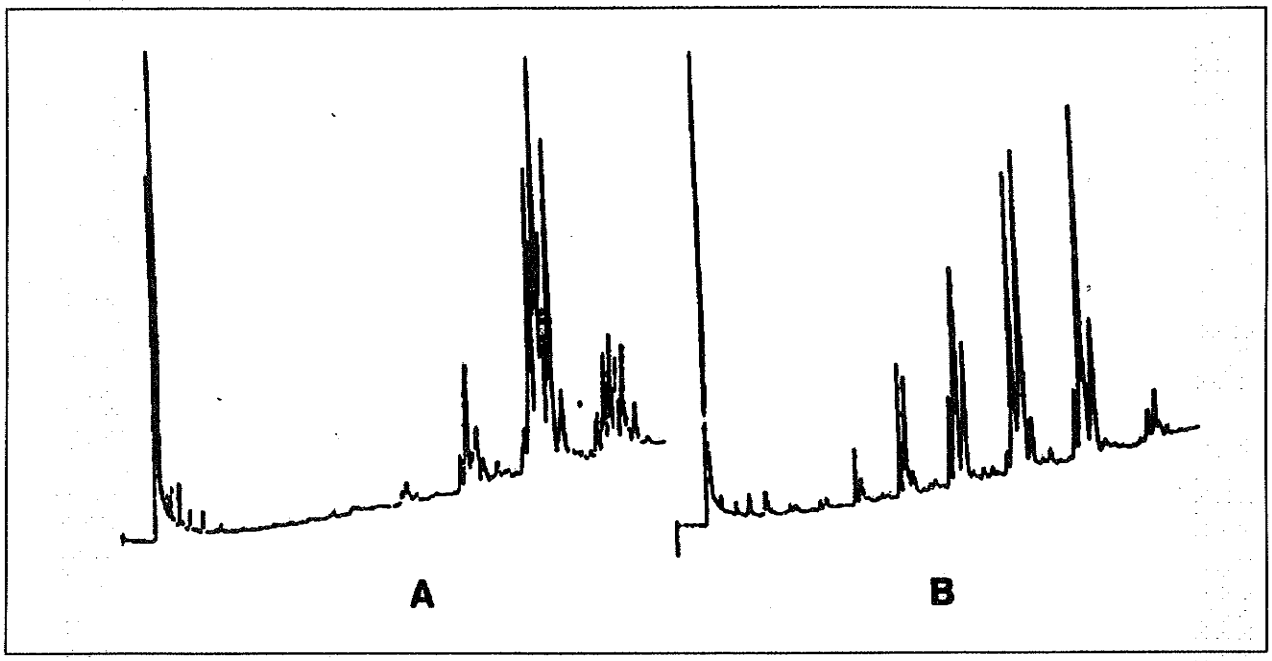

Fig. 2 Chromatograms of the triacylglycerols (TG) of pork fat (A) and of horse fat (B) (Geeraert *et al.*, 1986)

only found in the pig. The B/A ratio is an excellent parameter for the identification of pork in meat products.

Peptide analysis can also be performed after proteolytic degradation of meat products (e.g. with trypsine). The (unknown) peptides formed are then analysed with HPLC. In this way, addition of horse meat to canned meat products could be determined (Eckelmans, 1988).

4.4. Amino acid analysis

The total amino acid composition of a meat product can be determined with an amino acid analyser or a modified HPLC. When a meat product is hydrolysed to amino acids, the information of the secundary, tertiary and quaternary structure of the proteins, which can be species specific, is lost. Between beef, pork, sheep and fowl meat, no significant differences in the mean amino acid composition are found (with the exception of the methylhistidines (cf. 4.3.)). Horse meat contains significantly more histidine and less arginine (Ooghe *et al.*, 1985). Organ tissue can be differentiated from muscle tissue by its different amino acid composition (Ooghe *et al.*, 1985).

Free amino acids are present only in small amounts in meat and in non fermented meat products. Based on the composition of free amino acids, clearly separated clusters are found between beef, pork and chicken which can be recognized by pattern recognition techniques (De Baaij *et al.*, 1986). This method is also suitable for heated meat products. However, it should be born in mind that the free amino acid composition is prone to adulteration by addition of small amounts of synthetic amino acids.

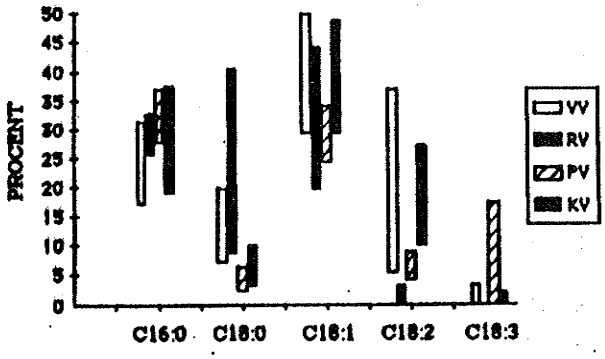

Fig. 3 Minimum en maximum values of the fatty acids in some species
VV = pork fat; RV = beef tallow; PV = horse fat; KV = hen fat

4.5. Species identification through analysis of fat

Species identification is also possible through analysis of the heat-stable fat fraction. The fat fraction is extracted from the meat product (e.g. with a mixture of chloroform - methanol 2:1, v/v) and is analysed. The most important analyses are carried out on the triacylglycerol (triglyceride) fraction (TG).

The isolated triacylglycerols can be separated by high temperature gas chromatography. The best separations are obtained on polar capillary columns. Figure 2 shows chromatograms of the triacylglycerols of pork and horse fat (Geeraert *et al.*, 1986). The difference between both chromatograms is obvious. Complete separation of all the triglyceride species, quantification and full identification of all the peaks in the chromatogram is still impossible at present time. With further improvement of the separating power of these high temperature columns, this technique may become very interesting.

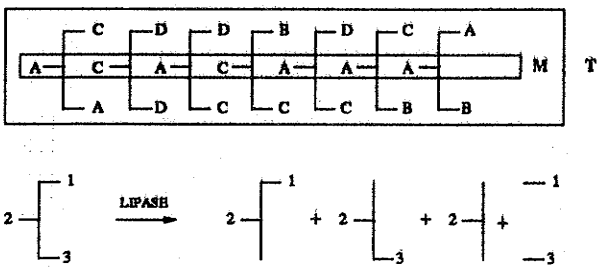

Fig. 4 Principle of the determination of the fatty acid composition in 2-position of TG

Some authors calculate species specific parameters from the fatty acid composition of the TG which can be determined by gas chromatography after conversion of the TG fatty acids to FAME's (Fatty Acid Methyl Esters). One of the best known examples is the recognition of horse meat on basis of 18:3n-3 (alphalinolenic acid) which is not, or only in small amounts, present in fats of other terrestrial animals. However, it is important to realize that the fatty acid composition of the TG of monogastric animals depends strongly on that of the fodder. Figure 3 visualizes minimum and maximum values of some selected fatty acids in the economically most important species.

In our laboratory a method was developped, extending fatty acid compositional data of the total triacylglycerols with those present in the 2-position. The prin-

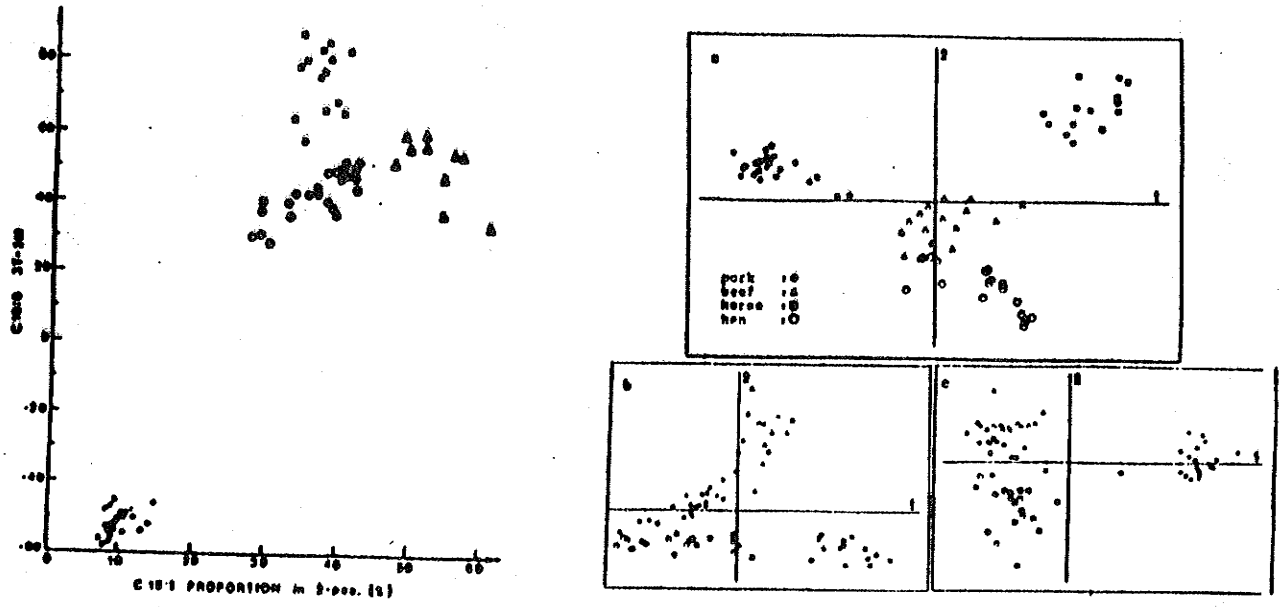

Fig. 5 Examples of pattern recognition graphs

ciple of the determination of the composition of the fatty acids in the 2-position of triglycerides is shown schematically in figure 4.

By an enzymatic TLC method, the 2-monoacylglycerols (monoglycerides) derived from TG by specific enzymatic removal of fatty acids present in the external positions of the TG, are isolated. These 2-monoacylglycerols (M) contain the fatty acids incorporated in 2-position (ß-position) of the triacylglycerols. By combination of the fatty acid composition in 2-position (M) and in the total triacylglycerols (T), species specific parameters could be obtained. Such parameters may be combined in bi-dimensional graphs in which different species appear as different clusters. Determination of the most optimal parameters was carried out with a pocket calculator (Verbeke and De Brabander, 1980 & 1985). Analysis is done graphically or by computer by pattern recognition using two different correlation graphs (Verbeke *et al.*, 1986; Thielemans *et al.*, 1989). An example of both graphs is given in figure 5.

Species identification of an unknown sample is carried out as follows. After TLC and GC analysis, all required parameters are calculated. This is carried out with a computer program developped in our laboratory (De Brabander, 1988). The parameters of the unknown is then plotted on the graph nr 1 which gives the best separation between pork fat and the other fats (see figure 5). Then there are three possibilities :

1. The point falls within the cluster of pork fat and the identification is completed.
2. The point falls within the cluster of one of the other fats. Then, the parameters which give the best separation between "beef tallow" versus "hen fat, horse fat" and "hen fat-horse fat" are used. If the point falls within the cluster of one of the pure fats, the result is known. With a computerprogram (De Brabander, 1988) it can be calculated objectively if the point falls within the 95% (or 99%) confidence ellips of the pure fat.
3. The data point falls between two clusters. Then it represents a mixture derived from more than one species. Here we will restrict ourselves to the procedure to be followed for a binary mixture. The relative amount of both fats can then be calculated from the relative distance from the data point to the centre of the two clusters. Obviously the value thus obtained is only an estimation. With a suitable computer program, the mean composition of the most prevalent fat in the mixture can be substracted from the mixture data. The program is carried out for three points in the neighbourhood of the estimated composition of the mixture. This calculation results in 3 fatty acid compositions for both T and M of the least prevalent fat. When the appropriate parameters of this "secundary" fat are plotted on the corresponding graphs, a straight line appears which cuts the cluster of the least prevalent fat. Thus, the identity of the least prevalent fat is obtained as well as an estimation of its fraction in the mixture.

4.6. Mass spectrometry

Heavy and expensive analytical equipment such as mass spectroscopy has been evaluated for species identification (Puckey and Jones, 1984). For this technique, the meat extracts are introduced into the mass spectrometer by a "direct probe insert". Thereafter, they are thermically degraded over a constant temperature interval (e.g. 20-350°C). The ions generated were found to be identical for all meat species investigated. Qualitatively, no differences could be found between the mass spectra of the different species. The only differences between the different species was found in the ratio of intensity of some ions. Using pattern recognition, different clusters for the different species were found.

5. CONCLUSION

This paper reviews the analytical methods used for species identification. The list of methods may not be exhaustive. However, it shows clearly that there is no such thing as an "all round method" for species identification. The methods applied vary from very simple and cheap (e.g. anatomical recognition) to very complicated and expensive (e.g. mass spectrometry). The exact problem to be solved and the nature of sample available dictates which method is most indicated. When two methods are equivalent from a scientific point of view, the price and speed of analysis play an important role. For fresh meat and unheated meat products, immunological methods are often the "methods of choice" when specific antibodies against the species to be determined are commercially available. However, this is not always the case. When a large number of samples are to be analysed, or a given sample is to be tested for many species, iso-electric focusing may be the method of choice. This technique is also valuable (and often necessary) for the identification of minor species. For strongly heated meat products, other chemical methods are indicated. These methods are mostly restricted to identification / determination of the four more important species (e.g. beef, pig, chicken and horse).

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SUMMARY

In the meat industry and in the meat trade, there is a growing interest in simple and cheap methods for species identification. The most important reasons are the difference in price and the economical regulations for meat of various species. Speciation may also be demanded for religious and ethical reasons.

The determination of the relative proportion of several species in a meat product represents a complicated analytical issue. The methods used for species identification can be divided into three groups : the anatomical and histological methods, the immunological methods and the physico-chemical methods. For the demonstration of the presence and the quantification of a certain species in a compound product, chemometric methods as pattern recognition are often used.

In this paper the different methods in use for species identification are reviewed. Emphasis is put on the determination of species specific proteins and fats in meat products.

SAMENVATTING

In de vleesindustrie en in de vleeshandel is er een groeiende belangstelling voor goedkope en eenvoudige methodes voor speciesidentificatie. De belangrijkste redenen zijn het verschil in prijs en de economische regulaties voor vlees van de verschillende species. Species identificatie kan ook worden gevraagd om religieuze of ethische motieven.

De bepaling van de relatieve proportie van verschillende species in een vleesprodukt is een ingewikkeld ana-

lytisch probleem. De methoden voor species identificatie kunnen in drie groepen worden onderverdeeld : de anatomische en histologische methodes, de immunologische methodes en de physico-chemische methodes. Om de aanwezigheid en het gehalte van een bepaald species in een samengesteld produkt aan te tonen worden vaak chemometrische methodes zoals patroonherkenning gebruikt.

In dit artikel wordt een overzicht gegeven van de verschillende methodes welke worden gebruikt voor species identificatie. De nadruk wordt gelegd op de bepaling van species specifieke eiwitten en vetten in vleesprodukten.

RÉSUMÉ

Il existe un intérêt croissant dans l'industrie et le commerce de la viande pour des méthodes simples et peu coûteuses mises au point pour l'identification des espèces animales. Les causes principales sont la différence de prix et la régulation économique des besoins en viande des différentes espèces animales. L'identification de l'espèce animale peut aussi être demandée pour des motifs religieux et éthiques.

La détermination des différentes proportions d'espèces animales dans un produit de viande est un problème analytique complexe. Les méthodes d'identification peuvent être subdivisées en trois groupes : méthodes anatomiques et histologiques, méthodes immunologiques et méthodes physico-chimiques. Souvent l'identification et la composition en espèces animales d'un produit composé sont réalisées par des méthodes chemométriques telles que la carte spectrale de reconnaissance.

Dans cet article sont décrites différentes méthodes appliquées pour l'identification des espèces animales. Les auteurs mettent l'accent sur la détermination des espèces, des protéines spécifiques et des matières grasses des produits de viande.