Comparison of the Results of the ELISA, Histochemical, and Immunohistochemical Detection of Soya Proteins in Meat Products

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Abstract

Pospiech M., Tremlová B., Renčová E., Randulová Z., Řezáčová Lukášková Z., Pokorná J. (2011): **Comparison of the results of the ELISA, histochemical, and immunohistochemical detection of soya proteins in meat products**. Czech J. Food Sci., **29**: 471–479.

This work compares the commonly used immunochemical methods for soya protein detection and alternative microscopic methods. Immunochemical methods were represented by the competitive ELISA method. Histochemical and immunohistochemical methods were used for microscopical examination. From a group of 252 meat products, each sample was examined for soya proteins by ELISA, histochemical, and immunohistochemical methods. The products came from the following categories: cooked sausages, ham, dry cooked sausages, and fermented sausages. The results showed that the highest accuracy was achieved by immunohistochemical examination. However, in the category of cooked sausages, this result was not statistically significant. Since the results in the individual categories differed, our results demonstrate that one single method does not always provide reliable and completely objective results. Immunohistochemical methods seem to be the most suitable for the verification of the reference immunochemical method results and prevention of false results.

Keywords: ELISA; allergen; immunochemistry; immunohistochemistry; histochemistry

Nowadays, we can often encounter plant protein additives in meat products.

Worldwide, plant protein sources are represented by 26% wheat, 18% corn, 15% rice, 15% soya, and 26% other plant protein sources (Gayer 1999). The reasons for substituting plant proteins for animal proteins are mainly of economic nature. However, sometimes this procedure improves technological parameters of the product or is related to the rationalisation of diet, dietetic value decline, fat limitation etc. (Pipek 1998). Among plant raw materials, soya has positive technical and sensorial properties. We can detect soya protein additive in meat products not only in the case of finely ground meat products, such as sausages and pâtés, but also in some roughly comminuted products. One usually cannot recognise the individual components of the products macroscopically, and the plant additive can be easily hidden by staining and flavouring with other additives

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(Ambrosiadis & Vareltzis 1998). Soya proteins are added into meat products in various forms, differing from one another in protein content percentage and the level of refining. Nevertheless, plant protein additives also include aspects with a negative influence on the consumer. It is mainly the adulteration which could represent a great risk for the consumer, especially in relation to the choice of unsuitable food for individuals with possible allergic reactions when the addition of soya is not labelled. The basic requirements for food labelling are included in EU legislation, as well as national legislation (Directive 2000/13/EC 2000; Decree 113, 2005).

The list of plant-originated raw materials added to meat products and possibly endangering consumer's health according to European Parliament and Council Directive 2003/89/EC as well as according to Czech legislation Appendix No.1 to Decree No. 113/2005 of Coll. includes soya as a potential allergen. National legislation also imposes requirements limiting the plant protein use in some meat product types due to the reasons for production quality preservation. Therefore, it is necessary to have methods which can detect these components. Plant protein detection is more complicated because of low concentrations of plant proteins used and because of the fact that soya protein structure can be modified by the production conditions.

Soya allergens can be detected in food products by analytical methods based on the detection of accompanying substances as reported by Belloque *et al.* (2002). These methods include microscopic, chemical, and biochemical ones. More accurate are the analytical methods based on the direct detection of soya protein or its allergic component. The direct methods include electrophoretic, immunochemical (immunodiffusion techniques, immunohistochemical method, immunoblotting, Western Blot, Dot-Blot, ELISA), and chromatographic methods (High-Performance Liquid Chromatography – HPLC, Ion-Exchange Chromatography, determination of Amino Acid Composition) (Belloque 2002). HPLC methods can be used as an alternative for the official ELISA methods. Castro-Rubio *et al.* (2005) describes HPLC with a high detection limit of 0.07% (w/w), and for quantitative examination with the detection limit of 0.2 % (w/w). In the Czech Republic, the immunochemical methods are often used in veterinary inspection, particularly the ELISA method is the most common. It offers a procedure suitable for qualitative findings and quantitative estimation of soya proteins in various food products (Strahle & Roth 1996). Some authors describe other ELISA methods. For example, Sakai *et al.* (2010) developed a fast sandwich ELISA kit for the determination of whole soyabean protein in processed foods. Another author described a kit for both qualitative and quantitative detection of soya protein (Fukal 1991). In order to determine other soya allergens, ELISA methods were developed for the detection of soya-trypsin-inhibitor (Kuhlhoff & Diehl 2004) and oil-body associated protein p34 (Morishita *et al.* 2008). Besides ELISA, immunological methods also include light scattering detection using gold nanoparticles (Sánchez-Martínez *et al.* 2009).

In relation to the microscopic methods, various staining methods were used for soya identification in meat products according to the information in literature: Gömöry's staining modified according to Grocott or trichrome staining according to Charvata (Heckmann *et al.* 1992), Bauer-Calleja, PAS in combination with light green or Procion brilliant blue (Coomaraswamy & Flint 1973). Other staining techniques include toluidine blue for metachromatic detection of texturised soya proteins (Flint & Meech 1978). However, plant protein additive presence is difficult to distinguish in spite of various staining methods existence due to various shapes (spongy-shaped, sickle-shaped, moon-shaped, or circular) corresponding to individual protein types. All the above-mentioned staining methods are mainly based on the detection of the accompanying polysaccharide structures of soya cells. This employs a technique of changing the polysaccharides present by means of oxidation of 1,2-glycol groups into unconjugated aldehyde groups, most often by utilising periodic or chromic acid. Polysaccharides modified in this way are able to create functional groups with staining agents by means of aldehyde reactions. The most common is Schiff's reagent which causes polysaccharides to turn pink up to purple-red.

With the traditional staining procedures, soya protein is stained similarly as muscle fiber proteins (Tremlová & Štarha 2002). With light green and Procion brilliant blue, muscle proteins and flour proteins are stained in the same way. However, soya protein is stained with a lower intensity than other proteins (Coomaraswamy & Flint 1973). Light microscopy allows for reliable, reproducible, and well documented evidence for plant protein additives presence, if additives of corresponding

size occur in the product. Histological evidence is of equal importance as electrophoretic and immunologic methods (Horn 1987). Concerning soya flour detection in the meat products, Feigl (1993) described a modified staining Bauer-Calleja method in his paper. Nevertheless, clear evidence for soya flour is only the detection of the so called structural elements, such as palisade and goblet cells (Horn 1987).

Another possibility to detect soya protein by histological methods is represented by the methods based on the accompanying substances of the plant additives, such as the detection of oxalate crystals. The amount of oxalate crystals which can be expected depends on the processing of the plant additives. While a lot of oxalate crystals occur in texturised soya preparations and in soya flour, soya protein concentrates include only a small amount of them, and in soya isolates these are only sporadic (Horn 1987).

In many medical fields, immunohistochemical methods combine the advantages of the traditional histochemical methods with sensitive immunological methods. Immunohistochemical methods for soya detection are rarely used in food analysis. On the other hand, immunohistochemical methods for the determination of bovine brain tissue are described by Wenisch *et al.* (1999), Tersteeg *et al.* (2002). As stated by Heitmann (1987), in the case of immunofluorescent examination is it possible to detect soya protein additive already from 0.1% onwards. Other authors focusing on food product analysis by immunohistochemical methods are also Boutten *et al.* (1999) who described a quantitative detection of soya protein in liver pâté in the range of 0.05% to 0.5% using immunohistochemical technique based on peroxidase – anti-peroxidase complex (PAP) in combination with image analysis. As declared by Boutten *et al.* (1999), immunohistochemical methods described in preliminary studies include immunohistochemical analysis with gold amplified by silver (Humbert *et al.* 1994), immunohistochemical analysis of anti-peroxidase system, and the above mentioned immunohistochemical PAP technique (Mason *et al.* 1982) which was applied by Boutten *et al.* (1999) in their work. Nowadays, more common are the methods based on avidin-biotin complex (ABC) due to a more powerful strengthening of the signal (Lukáš *et al.* 1997). One of the main advantages of immunohistochemical methods is the detection of soya directly in the histological sections. This technique can also be valuable for studying other proteins, for example collagen, as well as for the concentration and localisation in the product in combination with image analysis (Belloque *et al.* 2002). In the case of soya protein, it can be supported by the detection of complete soya protein or some of its allergic components. But, as reported by Kmínková *et al.* (2007), commercially available antibodies are only those against trypsin inhibitor and complete soya proteins. Their use as well as the use of antibodies developed in our institute for soya protein detection in model samples was published in the paper by Pospiech *et al.* (2009).

In this work, all of 252 meat products were examined for whole soya protein. For the purpose of comparison of the results of histochemical (hematoxylin – eosin staining and PAS Calleja staining), immunohistochemical, and ELISA examinations performed on all of 252 meat products, we processed the results statistically. All examinations were qualitative.

MATERIAL AND METHODS

Meat product sampling was done in the retail market, where the products were ready for sale. The samples were in the same form as they are supplied – in the market, vacuum-packed, wrapped in protective atmosphere or frozen. Four samples were taken from all products, and were subsequently adjusted to the size of 1 cm^3 and put into fixation solution (10% formalin). The samples were taken so that they represented the whole meat product, therefore the parts of the samples were as far from each other as possible – the outer part and the centre of the products. At sampling from the outer part, significant attention was concentrated to taking the under-wrap layer or the wrapping if it was a component of the food product.

A clear list of all examined meat products of the individual groups is presented in Table 1. Out of the examined meat products, 117 meat products belonged to the category in which soya protein additives are prohibited by the Czech legislation, for example cured fermented sausages (Decree 386 2001), 129 meat products fell into the category in which soya protein additives are not prohibited, such as cooked sausages, and six product represented the category of "standard" ham. All these meat products were bought randomly in the

Table 1. Detection of soya protein in meat samples

Sample type	Number of analysed samples	Number of soya protein additives detected*
Cooked sausages	129	114
Hams	17	3
Dry cooked sausages	37	7
Fermented sausages	69	3

*in the case of two positive results from all methods used

Czech market. The samples were processed in the histological laboratory for food histology of the Department of Vegetable Foodstuff and Plant Production, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno. If not specified otherwise, all chemicals and reagents used were bought from RNDr. Jan Kulich s.r.o. (Prague, Czech Republic).

The samples (1 cm^3) for histochemical and immunohistochemical examination were fixed in 10% aqueous solution of neutral formol for 24 hours. After fixation, the samples were dehydrated in a set of increasing concentrations of alcohol (30–96%) and acetone in Autotechnicon AT-4 and assembled into Paraplast blocks after preceding xylene baths (pure and anhydrous) which were then cut on a rotation microtome Leica RM 2255 (Leica Microsystems GmbH, Wetzlar, Germany). The sections were stretched in a water bath (40°C) and mounted onto microscope slides SuperFrost Plus (Menzel-Gläser, Menzel GmbH & Co KG, Braunschweig, Germany). Each sample was represented by four paraffin blocks from which microscopic sections were cut of 4 µm thickness, always with 50 µm intervals. The number of sections was chosen according to the variability caused by systematic impartial choice for histological examination (CE, coefficient of sampling error). Gundersen's and Jensen's method (1987) was used. We examined 12 sections at 40× and 100× magnifications with a light microscope Nikon ECLIPSE E200 (Fuji Bldg, Tokyo, Japan).

Histochemical examination

In histological sections, hematoxylin–eosin staining and PAS Calleja staining were used (Bancroft & Cook 2004; Manual 2005).

Hematoxylin–eosin. The sections were immersed in: (1) xylene, twice for 5 min; (2) absolute ethanol for 5 min, followed by 96% ethanol (v/v), 5 min; followed by (3) washing in water; (4) staining with Mayer´s haematoxylin – 1 g haematoxylin (RNDr. Karel Martyčák ML Chemica, Troubsko, Czech Republic), 1000 ml distilled water, 50 ml ethyl alcohol, 50 g potassium alum, 0.2 g sodium iodate, 1 g citric acid, 50 g chloral hydrate, for 5 min; (5) brief washing in water and diferentiation in acid-alkohol; (6) thorough washing in water for 10 min; (7) staining with eosin solution – 5 g eosin (RNDr. Karel Martyčák ML Chemica, Troubsko, Czech Republic), 5 g potassium bichromate, 100 ml absolute ethanol, 100 ml picricum acidum, 800 ml distilled water for 5 min; (8) quick washing in water and differentiation in 80% ethanol (v/v); (9) dehydration in 96% ethanol (v/v) for 3 min followed by absolute ethanol for 5 min; (10) clearing in xylene twice for 5 min; (11) mounting by drop of solacryl after which a micro coverslip was laid onto each section.

PAS Calleja. The sections were immersed in: (1) xylene twice for 5 min; (2) absolute ethanol for 5 min, followed by 96% ethanol (v/v) for 5 min; (3) washed in water; (4) treated with 0.5% solution of periodic acid dissolved in 96% ethanol (v/v) for 10 min; (5) rinsed well in reducing bath – 10 g potassium jodide, 10 g sodium thiosulfate, 10 ml 1M hydrochloride acid, add to 500 ml 70% ethanol (v/v); (6) rinsed well in 70% ethanol (v/v); (7) treated with Schiff's reagent – 1 g basic fuchsin (Penta, Chrudim, Czech Republic), 200 ml distilled water, 20 ml 1M hydrochloric acid, 2 g sodium metabisulphite, 2 g decolourising charcoal for 10 min; (8) washed in running tap water for 15 min; (9) stained with nuclei red – 10 g aluminium sulphate, 0.1 g nuclei red (RNDr. Karel Martyčák ML Chemica, Troubsko, Czech Republic), 100 ml distilled water for 15 min; (10) washed in water; (11) stained with Calleja solution – 100 ml 1% aqueous indigocarmine (v/v) (Merck, Darmstadt, Germany), 200 ml picricum acidum for 5 min; (12) washed in water; (13) dehydrated in 96% ethanol (v/v) for 3 min followed by absolute ethanol for 5 min; (14) clarified in xylene twice for 5 min; (15) mounted by a drop of solacryl after which a micro coverslip was laid onto each section.

Immunohistochemical examination was performed according to the procedure which had been successfully applied by Pospiech *et al.* (2009).

Immunochemical examination was performed according to the procedure which had been successfully applied by Renčová and Tremlová (2009).

Confirmatory results

The results, based on the hypothesis that a sample containing soya protein is evaluated positively, in at least two cases with any diagnostic method, were used as confirmatory results. In the case of two negative results, the sample was evaluated as negative. The results were evaluated qualitatively with signs +, –, and for dubious ones +/–. In the case of dubious result, the product was evaluated as both + and – in the statistical assessment.

Statistical analysis

The data were processed by mathematical – statistical methods using the software Unistat 5.1. (Ing. Jan Hofbauer, Dr., Brno, Czech Republic) and MS Excel (Microsoft Corporation, Washington, USA). In the statistical analysis, the research focused on the total number of positive and negative samples (possibly dubious) in each examination method, their relative representation (expressed in %) in the whole group, next to the percentage of positive and negative samples in the individual categories of meat products. The multitude of the results (+, –, +/–) acquired by monitored testing methods were compared by means of McNemar's test (Hendl 2004) which evaluates the transparency of the differences detected between the tested amounts of occurrences utilising test criterion calculation of χ^2 (chi quadrate).

RESULTS AND DISCUSSION

For a better transparency and discussion, the results of the individual examinations were listed for the individual meat product groups. The reason for this is also the different technological processes influencing the ability to detect soya additives, especially those depending on the temperatures used and the subsequent denaturation of the thermolabile components of soya proteins. Another reason is the different legislative requirements for the individual meat product categories in the Czech legislation (Decree 326, 2001). For the summarised results of the individual examinations (Table 2 and Figure 1). Histochemical (hematoxylin–eosin staining and PAS Calleja staining), immunohistochemical, and ELISA methods were applied on all 252 meat products examined for soya protein in our work. The methods are described in more detail in the Material and Methods section.

Cooked sausages

This group includes products of similar technologies and raw materials. Cooked, roasted, and

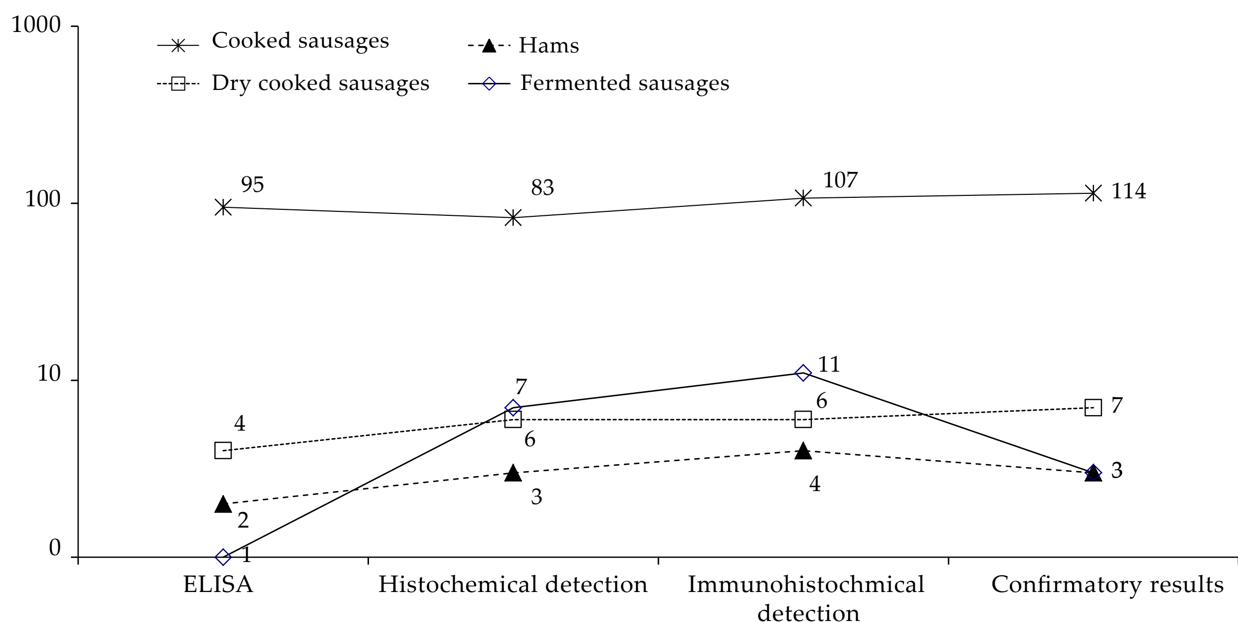

Figure 1. Soya proteins detected in meat products

Table 2. Comparison of the results obtained by histochemistry, immunohistochemistry and ELISA method

Sample type	Histochemistry	Immunohistochemistry	ELISA
Cooked sausages	χ^2 = 7.55*, P = 0.05	χ^2 = 23.06*, P = 0.01	χ^2 = 4.75*, P = 0.05
Hams	χ^2 = 1*, P = 0.05	χ^2 = 1*, P = 0.05	χ^2 = 1*, P = 0.05
Dry cooked sausages	χ^2 = 3*, P = 0.05	χ^2 = 0.33*, P = 0,05	χ^2 = 0.2*, P = 0.05
Fermented sausages	χ^2 = 2*, P = 0.05	χ^2 = 4*, P = 0.05	χ^2 = 0*, P = 0.05

*McNemar's

other meat products were represented in small quantities and their processing technologies are similar. For that reason, they are grouped together for the examination (Šedivý 1998). The cooking in the product core must be at least, at 70°C for 10 min (Decree 326, 2001). Frankfurters and sausages are most commonly consumed meat products and due to that, we chose a wide range of representatives of each type of these products. Soya protein was used in 114 products which is 88% (according to our hypothesis given above). However, only 21 products were declared on the package and 16 products were declared as undefined plant protein.

The divergence between the reference results and ELISA detection results was, according to McNemar's test, high because the final test criterion reached, χ^2 = 7.545, was higher than the critical limit χ^2 = 3.84 at the significance level of 0.05. That means that this method provided also different results in comparison to the reference results.

The divergence between the reference results and histochemical detection was, according to McNemar's test, very high because the final test criterion reached χ^2 = 23.06, was higher than one-sided critical limit χ^2 = 6.63 at the significance level of 0.01. That means that this method revealed very different results in comparison to the reference results.

The divergence between the reference results and immunohistochemical detection was, according to McNemar's test, very high because the final test criterion reached, χ^2 = 4.76, was higher than one-sided critical limit χ^2 = 3.84 at significance level of 0.05. That means that this method gave different results in comparison with the reference results, however, out of all three methods mentioned, these were the closest. Therefore, we can claim that this method is the most accurate although, according to McNemar's test, absolute conformity was not found.

The use of soya protein is not forbidden in the products of this group by legislation, which is also demonstrated by the high percentage of soya protein detected by our methods. The results for these meat products show that soya proteins have still been used in high quantities in this meat product category, which is in accordance with Pipek's statement (1998). Common use of soya protein is caused mainly by the economical aspect but, equally, also by favourable technological properties. Unfortunately, also in this group, where soya protein usage is not prohibited by the law, the producers do not observe other legal regulations directed at labelling soya protein additive as a potential allergen on the wrapping, in accordance with Directive 2003/89/EC (2003) and the Decree No. 113 (2005), and they also fail to label the processed raw materials which is regulated by the same Decree. Marking soya protein additives is essential, since soya represents a significant risk for the end consumer allergic to soya. Sporadically, even death caused by allergic reaction can occur in individuals allergic to soya after soya consumption.

Hams

In this category, the same results were achieved in comparison of the reference results with those obtained with all methods we used (ELISA, histochemistry, immunohistochemistry). The conformity between the reference results and those obtained with all our methods was, according to McNemar's test, high, because the test criterion achieved χ^2 = 1.00, was not higher than one-sided critical limit χ^2 = 3.84 at the significance limit of 0.05.

The results demonstrate that the examination in this category was very similar with all methods and, during testing, no significant differences at the significance level of 0.05 were found. The methods mentioned seem to be equally accurate for soya

detection in ham-like products. Positive findings occurred in 18% of this category, especially in three cases, out of which one was paradoxically ham of the highest quality, which is in contradiction with the valid legislation of the Czech Republic (Decree 326, 2001). The legislation does not allow the addition of soya or other plant proteins, other animal protein, fiber, starch, and staining substances to hams, except for the quality category "standard". Two other cases occurred in the category of "standard" ham. According to the valid legislation of the Czech Republic, the addition of soya protein is not prohibited in this category (Decree 326, 2001). However, one producer violated the regulation because he did not declare the presence of soya protein on the meat product package (Directive 2003/89/EC; Decree 113, 2005).

Dry cooked sausages

The conformity between the reference results and those of ELISA method was high according to the McNemar's test because the final test criterion achieved $\chi^2 = 3.00$, did not exceed one-sided critical limit $\chi^2 = 3.84$ at the significance level of 0.05.

The consensus between the reference results and those of histochemical method was high according to McNemar's test because the final test criterion achieved, $\chi^2 = 0.33$, did not exceed one-sided critical limit $\chi^2 = 3.84$ at the significance level of 0.05.

The consensus between the reference results and immunohistochemical method results according to the McNemar's test was high because the final test criterion achieved, $\chi^2 = 0.20$, did not exceed one-sided critical limit $\chi^2 = 3.84$ at the significance level of 0.05.

None of the tested methods demonstrated statistically significant difference from the reference results. Nevertheless, according to the achieved values of test criteria, one could estimate that the highest accuracy was achieved with immunohistochemical method and, paradoxically, the least accurate was the ELISA method.

In this category of meat products, national legislation (Decree 326, 2001) specifies the requirements for the composition of raw materials for the products Vysočina, Selský salám, and Turistický trvanlivý salám. It does not allow the use of fiber, mechanically separated meat, and mechanically separated poultry meat, plant or other animal proteins. Therefore, the fact that even in these meat products soya protein was detected in seven of them, which is 19%, is strange. In five products, soya protein presence was not marked on the meat product wrapping by which the producer also broke Directive 2003/89/EC (2003) and Decree 113 (2005). In two cases, the soya protein presence was declared on the package and demonstrated the producer's unawareness of his breaking the legal requirements for some dry cooked sausages products in Appendix No. 4 Table No. 5 (Decree 326, 2001). In one of them, the texturised soya protein was detected, which could be clearly determined by histochemical methods. In such a case, it is not necessary to perform immunohistochemical examination. This case indicates that it is appropriate to use histochemical method as the first choice since it is the cheapest and the fastest one of all the methods used.

Fermented sausages

In this meat product category, as in the previous one, our results demonstrated the addition of soya protein in three products, that is in four percentages.

The conformity between the reference results and those of ELISA method was high according to McNemar's test because the final test criterion achieved $\chi^2 = 2.00$, did not exceed one-sided critical limit $\chi^2 = 3.84$ at the significance level of 0.05.

The divergence between the reference results and histochemical method results was high according to McNemar's test because the final test criterion achieved $\chi^2 = 4.00$, exceeded one-sided critical limit $\chi^2 = 3.84$ at the significance level of 0.05. That means that this method provided different results in comparison with the reference results.

The consensus between the reference results and immunohistochemical method results was high according to McNemar's test because the final test criterion achieved $\chi^2 = 0.00$, did not exceed one-sided critical limit $\chi^2 = 3.84$ at the significance level of 0.05.

As indicated by the results achieved, immunohistochemical method seems to be more sensitive than the others while the least sensitive method is histochemical method.

Soya protein occurrence in this meat product category demonstrates the violation of legal requirements as well, probably because of the effort

to substitute for pure muscle protein (Coomaraswamy & Flint 1973). In these products, soya presence was not declared on the wrapping in any of the cases which puts the producer into contradiction to Decree 326 (2001). For the products in this category (Poličan, Herkules, Dunajská klobása, Lovecký salám, Paprikáš), the law also prohibits the use of fiber, machine separated meat, plant and other animal proteins (Decree 326, 2001).

Since the statistical results in the individual categories differed and those of immunohistochemical examination in cooked sausages presented in McNemar's test at the significance level of 0.05 divergence did not exceed the critical limit, it is not suitable to rely on one method only. It is better to test the products by two examination methods at least, in order to prevent false positive or false negative results. These results are in conformity with Fukal (1991), who described the problems with diagnostics between different kinds of soya protein and heated pretreatment of meat products.

CONCLUSION

The highest accuracy for all 252 products regardless of the category was achieved with immunohistochemical examination. The conformity between the reference results and those of immunohistochemical method was high according to McNemar's test because the final test criterion achieved $\chi^2 = 2.45$, did not exceed one-sided critical limit $\chi^2 = 3.84$ at the significance level of 0.05. The most common method of ELISA (Strahle & Roth 1996) revealed a high divergence from the reference results, according to McNemar's test, the final test criterion achieved $\chi^2 = 10.13$, exceeded one-sided critical limit $\chi^2 = 6.63$ at the significance level of 0.01. Histochemical detection results as well as those of ELISA method demonstrated a high divergence from the reference results according to McNemar's test, the final test criterion achieved $\chi^2 = 13.71$, exceeding one-sided critical limit $\chi^2 = 6.63$ at the significance level of 0.01. The results of McNemar's test for histochemical examination demonstrate that the detection of soya additives using this method is not easy, since clear evidence is understood as detecting structural elements of soya (Horn 1987) or described histological shapes of proteins. The exception is texturised soya protein with clear morphology. In this case, it is not necessary to use immunohistochemical examination.

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