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Immunofluorescence detection of pea protein in meat products

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ABSTRACT

In this study we developed an immunofluorescence method to detect pea protein in meat products. Pea protein has a high nutritional value but in sensitive individuals it may be responsible for causing allergic reactions. We produced model meat products with various additions of pea protein and flour; the detection limit (LOD) of the method for pea flour was 0.5% addition, and for pea protein it was 0.001% addition. The repeatabilities and reproducibilities for samples both positive and negative for pea protein were all 100%. In a blind test with model products and commercial samples, there was no statistically significant difference ($p > 0.05$) between the declared concentrations of pea protein and flour and the immunofluorescence method results. Sensitivity was 1.06 and specificity was 1.00. These results show that the immunofluorescence method is suitable for the detection of pea protein in meat products.

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Introduction

Plant seeds are important for human and animal nutrition (De Graaf et al. 2001; Sathe et al. 2006) and pea seeds are rich in proteins, fibre, minerals, vitamins and antioxidants (Alonso et al. 2001; Urbano et al. 2005). Pea proteins have a very high nutritional value because in comparison with cereals they contain a large amount of sulphur-containing amino acids. The major proteins of peas and other legumes are globulins and albumins; the globulin fraction contains primarily of 11S legumin and 7S vicilin (Mession et al. 2012). The sequence and structure of pea legumin are similar to soy legumin (Mills et al. 2004). Legumin is composed of an α -chain with a molecular weight of about 40 kDa that contains a large amount of acidic amino acids and a β -chain with a molecular weight of approximately 20 kDa that contains basic amino acids. These chains are linked by a disulphide bond into a complex with a molecular weight of 330 kDa (Lycett et al. 1983).

Thanks to its protein content, pea is widely used in both feed and food industries (Hulse 1994). Extending pea utilisation in the food industry was facilitated by the development of various protein concentrates, which can be used, for example, as protein supplements in bakery products, or as a substitute for meat in meat products (Tian et al. 1999, Tömösközi et al. 2001).

Pea proteins, however, may cause food intolerance and hypersensitivity (Sampson 2003). Allergy is one of the major health problems of contemporary man. This problem occurs to a greater extent in children (4–8%) due to their immature immune system and systems protecting their gastrointestinal mucosa. However, 1–2% of the adult population are affected by food allergies (Schäfer et al. 2001; Kim et al. 2011). In European countries, 0.8% of the population is allergic to pea (Sell et al. 2005; Mierzejewska et al. 2008). Pis S1 (vicilin) and Pis S2 (convicilin) globulins are mainly responsible for these allergies (Sathe et al. 2006). Malley et al. (1975) report that the molecular weight of pea allergen is 11 kDa, and state that only albumins give positive results in a skin test.

To determine exactly the minimum allergen doses capable of inducing allergic reactions is quite difficult in terms of both clinical diagnostics and food safety for the consumer. These doses are highly diversified and result from the biochemical and biological properties of allergenic ingredients. Exact determination of threshold doses is also difficult due to the fact that the allergenicity of products can be influenced by technological processes and the digestive process and absorption of food in the body (Jędrychowski et al. 2008). Detection of plant allergens is very complicated especially in complex matrices such as meat products

(Petrášová et al. 2014). The use of qualitative methods, i.e., methods detecting the presence or absence of a substance, is common in routine laboratory testing. They can be regarded as screening methods that are used before the more expensive examination by means of a quantitative method. The advantage is reduction of costs as well as time (Trullols et al. 2004). As with quantitative methods, users must be confident that the results of the qualitative method applied are suitable for the aimed purposes, which means that the method must be validated (European Commission 2002).

Commonly used methods for validation are based on quantitative methods, and there are many validation procedures accepted by the supervisory authorities and professional community working in the field (Trullols et al. 2004). Detection of pea proteins in meat products is discussed by Mellenthin and Galensa (1999), employing analysis of polyphenols using capillary zone electrophoresis, and by HPLC. If pisatin is detected in a meat product, the addition of a pea flour or protein is clearly proved. Unfortunately, pisatin is not a reliable indicating substance because it is not found in all pea protein samples analysed. As a phytoalexin, pisatin is only produced in a pea plant under certain environmental conditions. A qualitative 5-nuclease real-time PCR-based method for the detection of pea (*Pisum sativum*) in food is described by Brežná et al. (2006). In that work, the LOD of pea in meat pâtés was between 0.01% and 0.05%. It must be noted: regarding the empirical LOD observed in the model pâtés is that it is only achieved when at least 200 ng of DNA per reaction are available. This amount of amplifiable DNA cannot always be obtained from highly processed food samples (Brežná et al. 2006).

The immunofluorescence method is dependent on the detection of neither accompanying substances, such as pisatin, nor of DNA nucleotides; it directly detects pea proteins. Immunofluorescence detection of pea proteins (legumins and vicilins) in cotyledon parenchyma cell was researched by Craig et al. (1979). In order to facilitate effective monitoring of pea proteins usage in food products, it is necessary to develop an appropriate analytical method with a low LOD that should also be sufficiently rapid, specific and sensitive. The documented amount of pea flour or pea proteins added to a product may not be the only source of these materials. There might be also cross-contamination, where the amount of pea flour or protein cannot be estimated. This can also affect patients allergic to other legumes or peanuts (Richard et al. 2015). The aim of this study was, therefore, to develop a sensitive method for the detection pea proteins in meat products.

Materials and methods

Materials

Model meat products were used to determine the LOD, reproducibility and repeatability. Thirteen model samples were produced with the addition of Pea protein 80 (content: protein ($N \times 6.25$) $\geq 85\%$, fibre $\leq 1\%$, starch $\leq 4\%$, humidity $\leq 10\%$, pH 6.0–8.0) (Filip Degl, Czech Republic), and another 13 model samples with the addition of pea flour. Samples were manufactured from minced poultry meat with 0.001%, 0.01%, 0.10%, 0.50%, 1.00%, 1.50%, 2.00%, 2.50%, 3.00%, 3.50%, 4.00%, 4.50% and 5.00% of pea proteins and flour additions. These model samples were cooked in a water bath at 70°C (internal core temperature of the meat samples) for 10 min. Four blocks of 1 cm³ were taken from each sample and frozen. Using an HM 550 cryostat (Microm, Walldorf, Germany) these blocks were sliced into sections 4 µm thick. These sections were transferred to Thermo Superfrost plus slides (Thermo Scientific, Darmstadt, Germany). Two sections with trimming of 50 µm were cut from each block. Prior to use, the sections were left for 30 min at RT.

In order to determine the LOD, the above model products were used. To determine specificity and sensitivity, 12 products with declared composition were examined of which three samples were free of addition and nine samples contained declared additions of pea proteins. In order to determine the effectiveness of the method for detection of pea protein, results from samples of 53 products with declared compositions were examined by the McNemar test. A total of 49 products contained pea proteins or flour (samples bought from the market as well as model samples) and four samples were free of any addition.

Immunofluorescence detection

The immunofluorescence detection method was performed according to the following procedure. The method is based on the immunohistochemistry principle. Sections were immersed in: (1) 100% acetone twice for 5 min; (2) drying for 30 min at RT; (3) PBS, 80 g l⁻¹ NaCl, 2 g l⁻¹ KCl, 2 g l⁻¹ KH₂PO₄, 23.4 g l⁻¹ Na₂HPO₄ · 2 H₂O, 0.16 g l⁻¹ NaOH adjusted to pH 7.4 for 5 min; (4) 0.1 M Tris-HCl for 5 min at 650 W in a microwave; (5) cooling for 20 min at RT; (6) washed in PBS for 5 min; (7) 3% (v/v) H₂O₂ in PBS for 30 min; and then (8) washed in PBS twice for 5 min. The sections were then incubated successively: (9) for 30 min at 25°C with 5% (v/v) goat normal serum in PBS (Vector Laboratories, Burlingame, UK); (10) incubation with an anti-legumin mouse monoclonal IgG₁

(Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at 8°C diluted 1:1000 with antibody diluent (DakoCytomation ref. S0809, Glostrup, Germany); and (11) washed in PBS twice for 5 min; (12) incubation with 25 µl per section of anti-mouse IgG biotinylated antibody (Vector Laboratories, PK 6101) for 30 min at 25°C; and (13) then washed in PBS twice for 5 min; (14) incubation with Texas Red Streptavidin SA-5006 (Vector Laboratories) for 15 min and 25°C diluted 1:250 with PBS; and (15) washed in PBS twice for 5 min. The section were mounted with Vectashield mounting medium for fluorescence (Vector Laboratories) and a micro coverslip (Menzel-Gläser, Braunschweig, Germany) were laid onto each section. The chemicals and solutions used were obtained from RNDr. Jan Kulich s. r. o. (Prague, Czech Republic) and were used in p.a. quality.

Evaluation of the results

Within the microscopic examination, eight sections were examined for each sample at a magnification of 200× using a DM 3000 fluorescence microscope (Leica, Germany). Digitisation of samples was performed by means of a DFC 295 camera (Leica) and software Application Suite, Advanced Fluorescence 2.6.0.7266 (Leica). Sections were examined with the Texas Red filter which has a band-pass of 550–600 nm.

The obtained results were evaluated qualitatively with the symbols of – (Figure 1) for a negative result, ± (Figure 2) for a doubtful result and + (Figure 3), ++ (Figure 4) and +++ (Figures 5–7) for increasingly positive results. Within the immunofluorescence test, a doubtful result was considered a result in which antibody was not sufficiently bound to pea protein and was

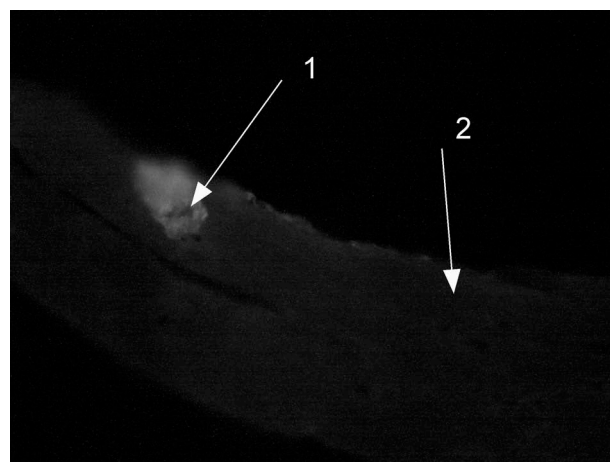


Figure 2. 0.001 pea protein concentration with ± intensity (200× magnification).



Figure 3. 0.001 pea protein concentration with + intensity (200× magnification).

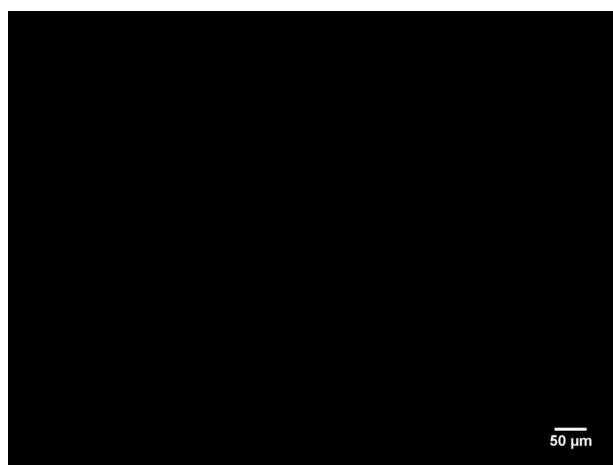


Figure 1. 0.000 pea protein concentration with – intensity (200× magnification).

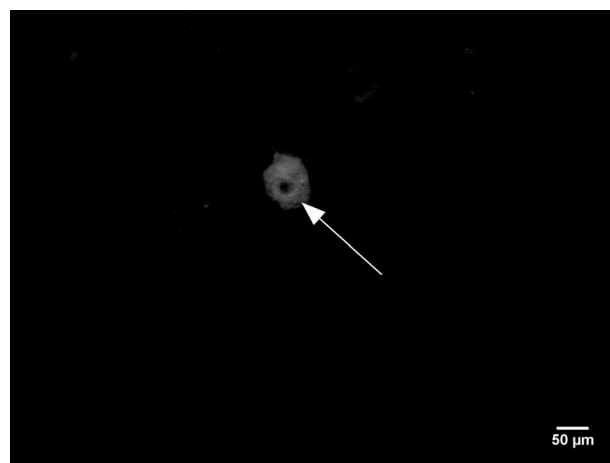


Figure 4. 0.001 pea protein concentration with ++ intensity (200× magnification).

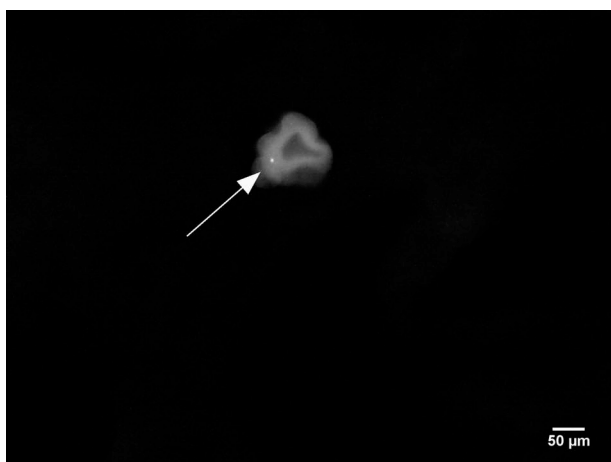


Figure 5. 0.001 pea protein concentration with +++ intensity (200× magnification).

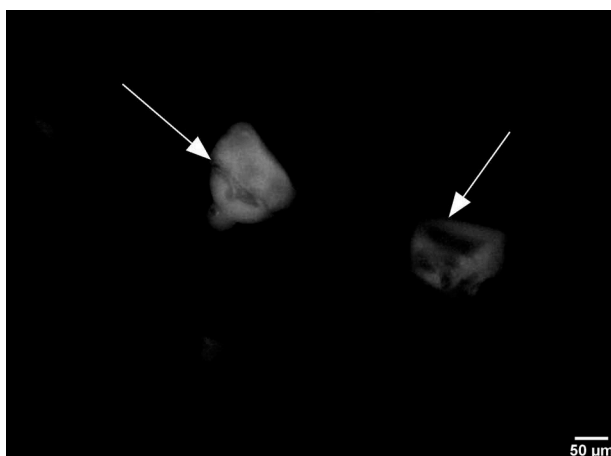


Figure 6. 0.01 pea protein concentration with +++ intensity (200× magnification).

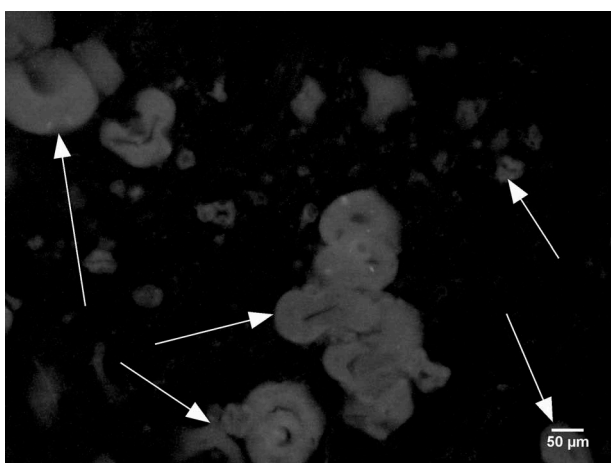


Figure 7. 1.0 pea protein concentration with +++ intensity (200× magnification).

difficult to be detected due to weak fluorescence intensity (arrow with the number 1 in Figure 2) or antibody was non-specifically bound on muscle fibre (arrow with the number 2 in Figure 2).

Repeatability and reproducibility

Repeatability was determined for positive as well as negative samples of pea protein according to the protocols (Suchánek 1999). Evaluation was performed by one examiner; samples and slides were prepared by a laboratory technician. The measurement was repeated 10 times each on different days.

Statistical analysis

Statistical analyses were performed using the Unistat program 6.1 for MS Excel (Unistat Ltd, 2012, Czech Republic). To evaluate the method, McNemar's test (Hendl 2004) was used to assess demonstrability of the differences found between the tested occurrences number by calculating the test criterion χ^2 (2×2 contingency table). Within the statistical analysis, numbers of positive and negative samples (doubtful samples were counted as negative and also positive) were qualitatively determined and their relative abundance was expressed as a percentage of the entire group of samples investigated.

Results and discussion

Signal intensity

Immunoreactivity was scored on a scale of – to ++++. The reactivity score was an average over all the model samples tested. The intensity for pea proteins in model samples for all concentrations was strong (+++). The intensity of fluorescence emission intensity from pea flour in model samples was strong for concentrations of 3.500–5.000%, moderate (++) for concentrations of 0.500–3.000%, and weak (+) for a concentration of 0.100%, while there was no signal found (–) for pea flour concentrations of 0.001–0.010%.

Detection limit (LOD)

The LOD for pea proteins was determined at 0.001% addition. For immunofluorescence detection of pea flour, it was determined as 0.5% addition (Table 1). The LOD is the first concentration value where determination is positive in all samples (100%). Usage of pea flour in meat products can be expected at about 2%, as

Table 1. Detection limit (LOD).

Model sample with concentration gradient (%)	Number of positive results (total = 8)	
	Pea flour	Pea protein
0.001	0	8
0.010	0	8
0.100	6	8
0.500	8	8
1.000	8	8
1.500	8	8
2.000	8	8
2.500	8	8
3.000	8	8
3.500	8	8
4.000	8	8
4.500	8	8
5.000	8	8

stated by Sun and Xiong (2015), as a 2% addition of pea flour in a meat product has a positive effect on its colour. These colour protections corresponded to improved oxidative stability of sarcoplasmic proteins and residual lipids, corroborating the remarkable radical-scavenging and reducing power of these pea products. Pea proteins and its hydrolysate proved to be capable of improving the oxidative stability of nitrosyl-hemochrome and enhancing the characteristic red colour in cooked nitrite-cured beef during refrigerated storage.

Repeatability and reproducibility

Repeatability is a useful parameter for qualitative methods. For immunohistochemical methods, this parameter is particularly important because the fixation of samples, sample processing and dyeing takes place on different days. This parameter is necessary to exclude the role of environment. The match was 100%.

The reproducibility parameter is used to exclude different ratings by different examiners. Examination was carried out by two examiners and the measurement was repeated five times. The match of examiners was 100%.

Sensitivity and specificity

The evaluation criteria for qualitative methods are sensitivity and specificity (European Food Safety Authority 2004). For qualitative methods, sensitivity means the ability of a method to detect truly positive samples as positive (O'Rangers & Condon 2000). The sensitivity rate is thus the probability for a given concentration, that the method will classify the test sample as positive (Massart et al. 1997). In contrast, specificity is defined as the ability of a method to detect truly negative samples as negative (O'Rangers & Condon 2000). The specificity rate is thus the probability, for a given

Table 2. Specificity and sensitivity of the immunofluorescence method.

Sample	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Declaration	N	N	P	P	P	P	P	P	P	P	P	N
Examination block A	N	N	P	P	P	P	P	P	P	P	P	N
Examination block B	N	N	P	P	N*	P	N*	P	P	P	P	N
Examination block C	N	N	P	P	P	P	P	P	P	P	P	N
Examination block D	N	N	P	P	P	P	P	P	P	P	P	N

Note: Values describe the differences between examination and declaration, including differences in blocks. S: sample; P: positive; N: negative; Declaration: declared content of pea flour and pea protein. *Differences between examination and declaration.

concentration, that the method will classify the test sample as negative (Massart et al. 1997). Specificity and sensitivity are dimensionless units that can be interpreted as a percentage. The diagnostic sensitivity is a fundamental feature of a qualitative method, which expresses its ability to detect small amounts of the analyte in a sample to produce the binary yes/no response at a predefined level of probability (Magnusson & Örnemark 2014). For this immunofluorescence method, sensitivity to pea proteins was determined at 1.06 (94.4%). Specificity was determined at 1.00 (100%) according to the protocols (Trullols et al. 2004) (Table 2). In S5 and S7 samples, false negativity was detected in block B, therefore it is appropriate to examine at least three blocks from each sample.

These results indicate that the method is highly sensitive and specific for the detection of pea proteins in meat products.

Expressing conformity

To express conformity between the immunofluorescence examination and the actual presence of pea proteins or flour, the statistical method of McNemar's test was applied. There was no statistically significant difference ($p > 0.05$) between the immunofluorescence method and the presence of pea proteins and flour (Table 3).

Table 3. 2×2 contingency table to compare immunofluorescence examination with the actual presence of pea.

	Truly positive	Truly negative
Immunofluorescence +	49	6
Immunofluorescence –	7	4
Chi-square statistic	0.0769	
Right-tail probability	0.7815	

This evaluation demonstrates that the method is suitable to detect pea proteins or flour in meat products and matches real additions.

Conclusions

An immunofluorescence method was developed to detect pea proteins. This analytical method has a low LOD, is also sufficiently rapid, specific and sensitive. The advantage of this method is elimination of false-positive results on the basis of knowledge of the morphology of pea protein. The method was validated based on the recommended parameters for qualitative methods. The LOD of the method was determined at 0.5% addition of pea flour and at 0.001% addition of pea proteins. Repeatability and reproducibility were determined to be 100% for both positive and negative samples for pea proteins. Moreover, in a blind test, the method was compared with the declared content, and there was no statistically significant difference ($p > 0.05$) between the immunofluorescence method and the label concentrations of pea proteins and flour. The results can therefore be considered as matching. Specificity and sensitivity of the method indicate that this immunofluorescence method is highly sensitive (1.06) and specific (1.00). The results point to the possibility of using this method for the detection of pea proteins in meat products.

Disclosure statement

No potential conflict of interest was reported by the authors.

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