

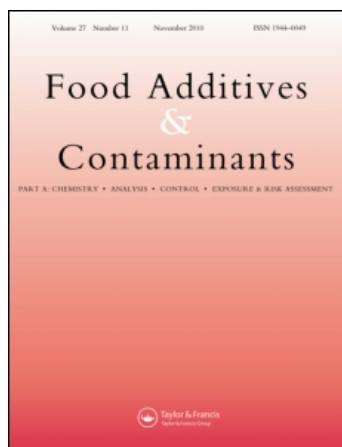
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Comparison of immunohistochemical, histochemical and immunochemical methods for the detection of wheat protein allergens in meat samples and cooked, dry, raw and fermented sausage samples

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Nowadays it is a common practice to add vegetable protein in the production of meat products. Because of the possible substitution of high-quality raw meat with vegetable protein without the labelling the product package and because of the allergenic potential of many vegetable proteins, it is important to develop accurate methods for its detection. The objective of the study was to compare histochemical, immunochemical (ELISA, ALERT gliadin screening test) and immunohistochemical methods for the detection of wheat protein in meat samples and sausages. Histochemical methods were useful for the detection of flour in meat samples, but the immunohistochemical method was better for the detection of wheat protein. ALERT gliadin screening test detected gliadin from 10 mg kg^{-1} , while an immunohistochemical method detected wheat protein concentrations from 1 g kg^{-1} and an ELISA method detected wheat protein concentrations from 4 g kg^{-1} . ALERT gliadin screening test showed results within 1 day, whilst an ELISA detection method took 2 days, and an immunohistochemical procedure took 5 days at the soonest, all including sample preparation. This study also focused on optimisation of an immunohistochemical method for samples of cooked sausage. In addition, three samples were sufficient for wheat protein detection at a concentration of 1 g kg^{-1} (and greater) with a confidence level greater than 95%.

Keywords: immunoassays; allergens; cereals; meat

Introduction

During food production, various plant-origin proteins are used as additives in order to improve the nutritional, flavour and visual properties of food products (Pickering et al. 1995). However, plant proteins can be major allergens that can cause an atopic eczema (Sicherer and Sampson 1999), gastrointestinal disturbances or other health complications in sensitive individuals (Hischenhuber et al. 2005). In the production of foodstuffs of plant as well as animal origin, common allergens include wheat gluten containing gliadin and prolamin proteins. These are present also in wheat flour (Day et al. 2006) and are very important for dough, pastry, biscuits and pasta too. Gluten-containing proteins in incorrectly labelled products can have a negative impact on the health of consumers suffering from celiac disease or gluten allergy (Ciclitira et al. 2005). The addition of plant proteins can negatively influence a guaranteed storage period of some meat products, since it increases the

water-binding capacity in foodstuffs (Vaňha et al. 2002). Plant proteins can also be used to substitute animal protein, which may cause, in the case of inadequate product labelling, food adulteration (Pospiech et al. 2009).

Wheat protein (gluten), wheat flour or breadcrumb, which also contains wheat flour, are due to technological and economical reasons traditionally added to meat products (especially to cooked sausage or forcemeat) and also to vegetarian foodstuffs production. Vegetarian foodstuffs production can also contain wheat germ. In addition, wheat protein can be contained in baking powder or other thickening agents.

The development of reliable and fast methods for the detection of plant-origin proteins, particularly of plant allergens, is essential (Poms et al. 2004). These requirements are met, for example, by these methods: immunochemical procedures (Moriyama et al. 2005) (radioimmunoanalysis, immunoelectrophoresis,

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enzymatic immunoanalytical methods – ELISA) (Asensio et al. 2008; Thompson and Méndez 2008), polymerase chain reaction (PCR) (Zeltner et al. 2009), and also immunohistochemical methods (Pospiech et al. 2009). Another possibility includes mass spectrometry (Šalplachta et al. 2005). Nevertheless, all these methods are not suitable for common food examination, in particular to meet the needs of the food control authorities.

The detection of allergens in food products can be very difficult as they are often present only in trace amounts or are masked by the food matrix (Poms et al. 2004). Immunological techniques are generally very sensitive and specific and have become very popular for the detection of small amounts of allergens. These methods based on the reactivity between the antigen and the antiserum recognise only particular regions of the protein with a characteristic structure called epitopes (Belloque et al. 2002). This mechanism is the same for immunohistochemical methods that we used to develop histological and highly sensitive immunological methods.

With regard to the fact that the amount of allergenic protein inducing allergic reaction shifts within a broad range of concentrations and is specific for each individual (Stern et al. 2001), an important monitored aspect is the reliability of detection of added wheat protein. The aim of this study was to compare histochemical, immunochemical and immunohistochemical methods for the detection of wheat protein in meat and sausage samples with and without additives.

Materials and methods

Preparation of meat samples

Meat samples were prepared from pork meat without additive (control) and with 1, 2, 3, 4, 5, 10, 25 and 50 g kg⁻¹ of wheat protein (Amylon, a.s., Havlíčkův Brod, Czech Republic). Other meat samples were prepared from pork meat with 5, 10, 25 and 50 g kg⁻¹ of wheat medium flour (Penam, a.s., Brno, Czech Republic). Another meat sample was prepared from pork meat with 10 g kg⁻¹ wheat protein and 10 g kg⁻¹ wheat soft flour (Penam).

Preparation of sausage samples

Sausage samples were manufactured based on recipes described by Šedivý (1998). The products included cooked sausage, raw sausage, dry sausage and fermented sausage without additive (control), and with 1, 5, 15 and 30 g kg⁻¹ of wheat protein (Amylon). The wheat protein was added during processing in a sausage cutter.

Sample treatment and preparation

For the ELISA method and ALERT gliadin screening test, samples of 250 g were taken from each meat and sausage sample and subsequently frozen. Other samples sized 1 cm³ were taken from meat samples. Samples sized 1 cm³ were taken from sausage samples of cooked sausage from six locations (at either end and in the middle of the product, always from the centre and subsurface layer). As the seventh sample of cooked sausage, homogenate was prepared from either end and from the middle of the product by means of grinding in a blender TM 4510 type FP 2111 (Concept, Hong Kong, China). The sample sized 1 cm³ from raw sausage, dry sausage and fermented sausage was only taken from the centre of model meat product.

Samples were processed for histological and immunohistochemical examination according to the procedure described by Pospiech et al. (2009). Samples (1 cm³) were fixed in 10% water solution of neutral formalin for more than 24 h. After fixation the samples were dewatered in ascending sequence of alcohol in the autotechnicon apparatus Leica TP1020 – Automatic Tissue Processor for Histology Applications (Leica Microsystems GmbH, Wetzlar, Germany) and embedded into paraffin blocks in Paraplaste that were cut into 4 µm sections on a rotation microtome Leica RM2255. Model meat product samples of cooked sausage were cut into 75 sections (25 slides with three sections each), while 50 µm were always trimmed between individual sections. The sections were spread on the surface of the water and mounted on slides SuperFrost plus (Menzel-Gläser, Menzel GmbH & Co KG, Braunschweig, Germany).

Immunohistochemical examination of samples

In total 117 sections from 13 meat samples (nine sections from each sample) were processed immunohistochemically. Further, 2625 sections of cooked sausage and six sections of every other sausage samples were examined immunohistochemically.

The procedure for the immunohistochemical detection of wheat protein based on avidin–biotin complex (ABC), derived from a procedure described by Pospiech et al. (2009), was used. This indirect three-stage method is more an amplification method which uses a high binding affinity between avidin and biotin for antibodies detection. At first a polyclonal anti-wheat antibody isolated from a rabbit (Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1 : 1000 diluted with antibody diluent (DakoCytomation ref. S0809, Glostrup, Denmark) is bound to an antigen. In negative control, the primary antibody was substituted by antibody diluent (DakoCytomation ref. S0809). In the second phase, a biotin-conjugated secondary antibody (VectorLaboratories, PK 6101,

Burlingame, CA, USA) is attached to the bound primary antibody. The third stage is bond between ABC–peroxidase from the ABC reagent (Vector Laboratories, PK 6101) and a secondary biotinylated antibody which significantly amplifies the signal.

The background was visualised in a Calleja bath (Calleja 1897) for 5 min or in a toluidine bath for 1 min and washed in the water bath while some sections were left without background staining. Meat samples containing 10 g kg^{-1} wheat protein as well as 10 g kg^{-1} wheat soft flour, after immunohistochemical detection, were stained by a combination of PAS–Calleja (Protocols 1997), Lugol–Calleja (Protocols 1989), Lugol (Bancroft and Cook 1994)–toluidine blue (Flint 1994), and PAS–toluidine blue staining (Bancroft and Cook 1994). For a summary of all used examinations, see Table 1.

- PAS–Calleja staining: sections were immersed in: (1) orthoperiodic acid for 10 min, (2) washed in a reduction bath and then (3) in 70% aqueous ethanol (v/v), (4) in Schiff's reagent for 15 min, (5) washed in a water bath for 15 min, and (6) in a Calleja bath for 5 min and washed in a water bath.
- Lugol–Calleja staining: sections were immersed in: (1) a Lugol bath for 5 min, and (2) in a Calleja bath for 5 min and washed in a water bath.
- Lugol–toluidine blue staining: sections were immersed in: (1) a Lugol bath for 5 min, and (2) in a toluidine bath for 1 min and washed in a water bath.
- PAS–toluidine blue staining: sections were immersed in: (1) orthoperiodic acid for 10 min, (2) washed in a reduction bath and then (3) in 70% aqueous ethanol (v/v), (4) in

Schiff's reagent for 15 min, (5) washed in a water bath for 15 min, and (6) in a toluidine bath for 1 min and washed in a water bath.

Histochemical examination of samples

From selected meat samples (meat with 25 g kg^{-1} addition of wheat protein, meat with 25 g kg^{-1} addition of wheat flour), 12 sections were stained by general microscopic staining (six sections with haematoxylin and eosin (Bancroft and Cook 1994), six sections with toluidine blue (Flint 1994)), 12 sections by special microscopic staining (six sections with Lugol–Calleja (Protocols 1989), and six sections with PAS–Calleja (Protocols 1997)). For a summary of all used examinations, see Table 1.

Examination procedure

After histochemical and immunohistochemical detection, a total number of 2766 sections were examined by a light microscope (Nikon ECLIPSE E200; Fuji Bldg, Tokyo, Japan) at a magnification of $40\times$. In the case of obscurities, magnifications of $100\times$ and $400\times$ were used. A set of digital photographs was captured to document the results by means of a digital camera (Canon PowerShot G9; Canon Inc., Tokyo, Japan) with the use of picture-capturing software PSRemote Version 1.5.2 (Breeze Systems, Bagshot, UK).

Immunochemical examination of samples

ELISA method

The indirect competitive ELISA was prepared according to Rencova et al. (2000) and Rencova and Tremlova (2009) and was then modified for the

Table 1. Summary of methods used for examination of sample groups.

Sample	Methods		
	Histochemistry	Immunochemistry	Immunohistochemistry
Meat sample with wheat protein	General staining (haematoxylin and eosin, toluidine blue), special staining (Lugol–Calleja, PAS–Calleja)	ELISA	+ Calleja, + toluidine blue
Meat sample with flour	General staining (haematoxylin and eosin, toluidine blue), special staining (Lugol–Calleja, PAS–Calleja)	–	+ Calleja, + toluidine blue
Meat sample with flour and wheat protein	–	–	+ PAS–Calleja, + PAS–toluidine blue, + Lugol–Calleja, + Lugol–toluidine blue
Sausage samples	–	ELISA, ALERT test	+ toluidine blue

Note: +, Preparation background staining; –, examination not performed.

detection of wheat gliadin in model meat samples. Meat samples with wheat protein were simultaneously tested for the presence of wheat proteins.

The immunisation antigen was prepared from purified wheat gliadin (Sigma-Aldridge, St. Louis, MO, USA). The amount of 5 mg of gliadin was dissolved in 1 ml of 70% ethanol v/v with the addition of 10 µl of acetic acid. This volume was adjusted to 5 ml with the protein concentration of 1 mg ml⁻¹. Three mice BALB/C, 10 weeks old and 22 g, were immunised using purified gliadin antigen dissolved in 70% ethanol mixed 1:1 with complete Freund's adjuvant (Sigma-Aldridge) administered subcutaneously. The antigen concentration was 50 µg ml⁻¹; the immunisation dose was 0.1 ml. Boosters were applied twice in 3-week intervals 1:1 with Freund's incomplete adjuvant. Test sera were collected by tail bleeding 10 days after each injection; the titre of the sera was then determined by indirect ELISA. The last booster was applied intraperitoneally 4 days before the fusion. Spleen cells were fused with the myeloma cell line (P3x63.Ag8.653., ATCC CRL 1580) at a ratio of 5:1 in the presence of polyethylene glycol. The purified antibodies were titrated against gliadin using indirect ELISA.

Meat samples of 20 g were processed in the blender with 200 ml of 70% ethanol v/v. After a 30-min extraction process, the extract solution was centrifuged at 10,000 g and 4°C for 10 min and the supernatant was used for analysis.

ALERT gliadin screening test

For the comparison of results achieved by the above-described procedures, sausage samples were examined also by the ALERT gliadin screening test (Neogen Corporation, Lansing, MI, USA) designed for qualitative analysis of gluten-free food products for the presence of gliadins and prolamins found in wheat, barley and rye. The producer claims that the test is able to detect already an addition of 10 mg kg⁻¹ of these allergens. The ALERT test is based on the principle of a sandwich enzyme-linked immunosorbent assay. Gliadin is first extracted from samples with a 40% dilution of ethanol. Extract is diluted in phosphate-buffered saline (PBS), 80 g l⁻¹ NaCl, 2 g l⁻¹ KCl, 2 g l⁻¹ KH₂PO₄, 23.4 g l⁻¹ Na₂HPO₄·12H₂O, 0.16 g l⁻¹ NaOH adjusted to pH 7.4, and applied to antibody-coated wells where gluten will bind to the antibody during an incubation period. Any unbound gliadin is washed away and a second antibody, which is enzyme labelled, is added. The detector antibody binds to the gliadin during another incubation period. Unbound antibody is washed away and a one-step substrate is added. Colour develops as a result of the presence of bound-labelled antibody. A blue colour indicates samples containing high levels of gliadin, while

purple or red samples contain little or no gliadin. Colour is compared with negative and positive control.

Statistical analysis

Statistical evaluation was performed by statistical software Minitab 15 (Minitab User's Guide 2. 2000. Data analysis and quality tools; State College, PA, USA). We used logistic regression analysis to find out how many sections from the sausage samples must necessarily be examined to be able to decide with a confidence of 95% that the wheat protein is present in the product.

Results and discussion

Special staining (Lugol–Calleja and PAS–Calleja) indicated the presence of flour on the basis of different colours on the microscope slide. Lugol–Calleja staining indicated the presence of flour starch particles in meat samples that were within dark purple and black (Figure 1), and PAS–Calleja staining highlighted all polysaccharides in pink. The staining results for meat samples were the same at different spiking levels of wheat flour. Other components of meat samples were evident as various tones of green. On the other hand, general staining (haematoxylin and eosin, toluidine blue) indicated flour only on the basis of their typical appearance and structure in the microscopic picture (Figure 2). Flour formed clusters containing particles of wheat starch that looked similar to small and large lentil-like kernels. A similar finding was reported by Tremlová and Štarha (2002). However, the shape of wheat starch can be changed after technological processing (Aguilera and Stanley 1990).

Wheat protein in meat samples was histochemically identified only on the basis of their typical spongy

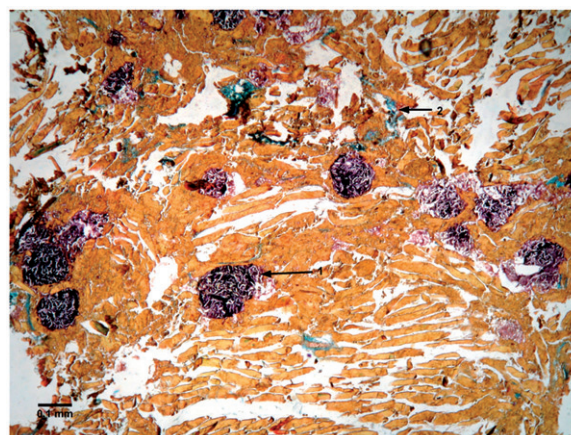


Figure 1. Meat sample – meat with wheat flour, Lugol – Calleja staining, examined at 40x, (1) wheat flour, (2) collagen.

structure with openings (Figure 3). Moreover, special staining of starch indicated sporadic starch granules contained in wheat protein. In contrast to flour, wheat protein was not significantly differentiated based on different colour from other components. Only general staining with toluidine blue gave plant-origin proteins colours in different shades of blue (wheat protein is light cyan) from other product components (Flint 1994). Simultaneously, wheat protein in meat samples was detected by immunohistochemical methods where wheat protein was visualised as brown particles by DAB (3-3'-diaminobenzidine) chromogene (Figure 4) in contrast to stained background with toluidine blue (blue) or with Calleja staining (green). The staining results for meat samples and sausage samples were the same at different spiking levels of wheat protein. However, this method had limits in detection of wheat flour, where this staining caused only minor changes (Figure 5). This phenomenon was probably caused by a relatively low concentration of wheat protein in wheat flour (70–130 g kg⁻¹ proteins) (Keletunç and

Breslauer 2003), so individual protein epitopes with conjugated antibodies were too far away from each other and did not present a colourful picture visible to one's eye.

Based on the above-described results, meat samples containing wheat protein as well as wheat flour were subjected to the combination of immunohistochemical and histochemical examination specialised on starch detection. Various methods for the preparation staining were used to stain backgrounds after immunohistochemical processing. Combinations used were PAS–Calleja, Lugol–Calleja and, for stronger contrast between wheat protein highlighted by DAB chromogene and other components, Lugol–toluidine blue and PAS–toluidine blue. From the above combinations, PAS–Calleja staining (Figure 6) provided the largest colour differences between the monitored components. Combinations with Lugol solution staining

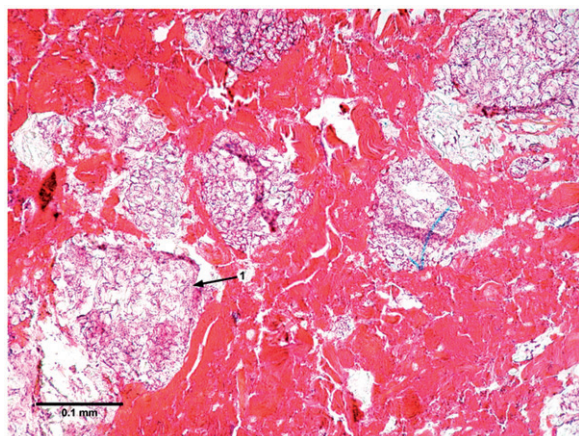


Figure 2. Meat sample – meat with wheat flour, Hematoxylin – Eosin staining, examined at 100x, (1) wheat flour.

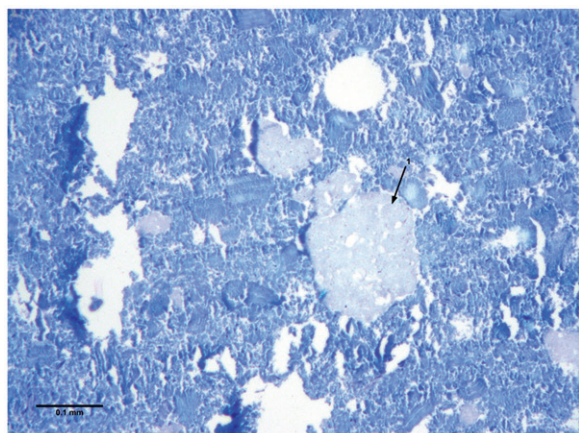


Figure 3. Meat sample – meat with wheat protein, toluidine blue staining, examined at 100x, (1) wheat protein.

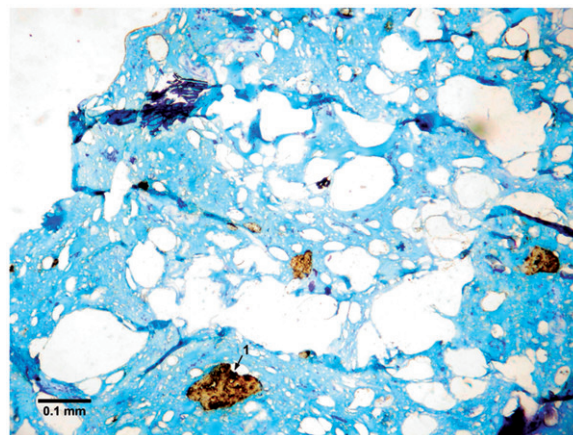


Figure 4. Sausage sample – cooked sausage with wheat protein addition of 15 g kg⁻¹, immunohistochemical method, highlighted by DAB chromogene, toluidine blue staining, examined at 40x, (1) wheat protein.

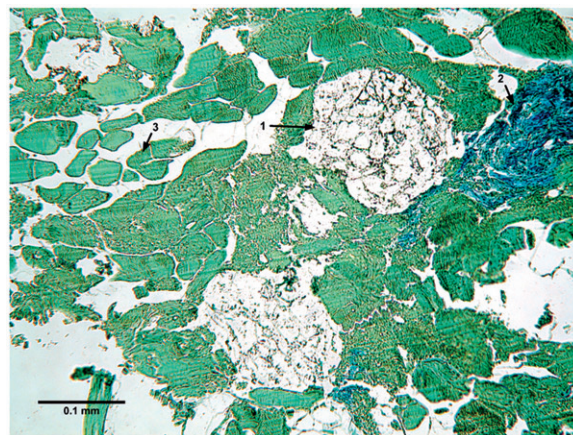


Figure 5. Meat sample – meat with wheat flour, immunohistochemical method, highlighted by DAB chromogene, staining according to Calleja, examined at 100x, (1) wheat flour, (2) collagen, (3) muscle tissue.

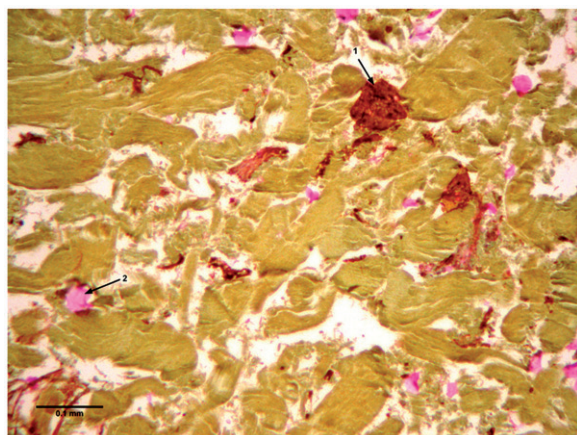


Figure 6. Meat sample – meat with wheat flour and wheat protein, immunohistochemical method, highlighted by DAB chromogene, PAS – Callea staining, examined at 40x, (1) wheat protein, (2) wheat flour.

Table 2. Comparison of ELISA method with immunohistochemical examination of meat samples.

Wheat protein addition (g kg^{-1})	ELISA	Immunohistochemical method
0	–	–
1	–	+
2	±	+
3	±	+
4	+	+
5	+	+
10	+	+
25	+	+
50	+	+

Note: +, Positive; –, negative; ±, dubious.

resulted in weaker colour contrast between starch and wheat protein and partially in interference of individual stains.

Immunohistochemical detection of meat samples was compared with results achieved by reference ELISA method. The ELISA method reliably detected wheat protein concentrations from 4 g kg^{-1} while immunohistochemical method detected wheat protein concentrations from 1 g kg^{-1} (Table 2). A generally acknowledged requirement on the detection limit for allergen is $1\text{--}100 \text{ mg kg}^{-1}$ of allergens in food products, depending on allergen type and possibly depending on analytical possibilities (Koppelman and Hefle 2006). The upper limit of this range is identical with the upper limit of an acceptable amount of gluten in gluten-free food products as stated by Commission Regulation (EC) No. 41/2009 (European Commission 2009) concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. It establishes that the retail-sale form of food products for people intolerant to gluten must not contain more than 20 mg kg^{-1} of gluten in case they do not include any constituents from wheat or other types of *Triticum*, or more than 100 mg kg^{-1} of gluten in case constituents from wheat or other types of *Triticum* were used at their production.

The detection of wheat protein by ELISA, the commercially available ALERT gliadin screening test, and the immunohistochemical procedure with toluidine blue staining in sausage samples is shown in Table 3. The only method with negative detection of samples with wheat protein was the ELISA method

Table 3. Comparison of the ELISA method, ALERT gliadin screening test and immunohistochemical examination of sausage samples.

Product	Wheat protein addition (g kg^{-1})	ELISA	ALERT gliadin screening test	Immunohistochemical method
Cooked sausage	0	–	–	–
	1	–	+	+
	5	+	+	+
	15	+	+	+
	30	+	+	+
Dry sausage	0	–	+	–
	1	–	+	+
	5	+	+	+
	15	+	+	+
	30	+	+	+
Raw sausage	0	–	+	–
	1	+	+	+
	5	+	+	+
	15	+	+	+
	30	+	+	+
Fermented sausage	0	+	–	–
	1	+	+	+
	5	+	+	+
	15	+	+	+
	30	+	+	+

Note: +, Positive; –, negative.

(Table 2 and 3). We suppose that this negative detection was possibly caused by decreased sensitivity of the detection method (detection limit = $4\text{--}5\text{ g kg}^{-1}$), or possibly in cooked products by the worsened availability of antigen after technological processing of the product. The only method that met the commonly acknowledged detection limit was the ALERT gliadin screening test. The producer of this test claims an ability to detect gliadin already from 10 mg kg^{-1} (20 mg kg^{-1} of gluten) in this test, which is confirmed also by the results of this study. However, in our control sausage samples of dry sausage and raw sausage without any wheat protein addition this test showed a false-positive reaction. This might have been caused by cross-contamination of samples during

preparation for examination, which included sample grinding (in one machine). Including sample preparation, the immunohistochemical procedure showed results in 5 days at the soonest, while the ELISA detection method showed results in 2 days, and ALERT gliadin test within 1 day.

The immunohistochemical method seems to be sensitive and reliable and it contains current possibilities of food microscopy – the detection of food components, quality detection, the arrangement of individual food components, the adulteration of foodstuffs, the detection of risk components in food products, and possible quantification of food components (Heertje and Leunis 1997). Microscopy represents an effective and in many cases irreplaceable instrument for achieving this

Table 4. Summary of immunohistochemical examination of sausage samples of cooked sausage.

Wheat protein additive	Sample	Number of sections with a positive result	Number of sections with a negative result	Number of examined sections	Losses during processing
1 g kg^{-1}	ZO	74	0	74	1
	PO	71	4	75	0
	KO	68	3	71	4
	ZS	73	1	74	1
	PS	64	5	69	6
	KS	64	9	73	2
	HO	63	12	75	0
5 g kg^{-1}	ZO	74	0	74	1
	PO	72	0	72	3
	KO	71	2	73	2
	ZS	73	0	73	2
	PS	75	0	75	0
	KS	75	0	75	0
	HO	72	3	75	0
15 g kg^{-1}	ZO	70	0	70	5
	PO	72	0	72	3
	KO	72	0	72	3
	ZS	69	3	72	3
	PS	72	0	72	3
	KS	71	0	71	4
	HO	72	0	72	3
30 g kg^{-1}	ZO	75	0	75	0
	PO	75	0	75	0
	KO	75	0	75	0
	ZS	75	0	75	0
	PS	75	0	75	0
	KS	75	0	75	0
	HO	73	1	74	1
Control	ZO	0	37	37	38
	PO	0	70	70	5
	KO	0	48	48	27
	ZS	0	63	63	12
	PS	0	65	65	10
	KS	0	60	60	15
	HO	0	68	68	7
Sum		2010	454	2464	161

Notes: HO, homogenate from various parts of the product; KO, product end, undercover layer; KS, product end, product centre; PO, product middle part, undercover layer; PS, product middle part, product centre; ZO, product beginning, undercover layer; ZS, product beginning, product centre.

necessary information. Unlike immunochemical methods that can mostly detect only one constituent in one detection, food microscopy allows for the detection of several food components within one analysis (Heertje and Leunis 1997).

This study was also focused on optimisation of the immunohistochemical procedure, which was performed in sausage samples of cooked sausage. We found out how many sections from the sausage sample are necessary for examination to be able to decide with a confidence of 95% that wheat protein is present in the product. If the test of dependence of wheat protein concentration with respect to the probability of detection (using logistic regression) is made, the wheat protein concentration will be significant at a significance level of 0.05. If the information that there are seven types of samples is added to the model, the factor 'sample type' will also be significant at a significance level of 0.05. It is a problem because a model would be made where wheat protein detection is examined with respect to concentration and sample type. But there are not enough data for this model. We focused on the sample with the worst detection ratio, which was the homogenate of cooked sausage with 1 g kg^{-1} addition of wheat protein (Table 4). The point estimate for the probability of wheat protein detection was $63/75 = 0.84$. If only one section was made, the probability of wheat protein detection was $p = 0.753692$ using a one-sided interval estimate with confidence of 95%. If two sections were made, the probability of wheat protein detection was $1 - (1 - p)^2 = 0.9393$, which was still less than 0.95. If three sections were made, the probability of wheat protein detection was $1 - (1 - p)^3 = 0.9850$, which was greater than 0.95. Therefore, three sections were sufficient for at least one wheat protein detection at a concentration of 1 g kg^{-1} (and greater) with a confidence level greater than 95%. The calculated minimum number of examined sections necessary for reliable detection then needs to be increased by losses caused by harm to sections during processing (e.g. a section drifting away while staining), which were 6.1%. Optimisation also included shortening the time of the immunohistochemical method. In order to decrease time consumption, this procedure was shorted to 1 day. The main change was especially in incubation with a polyclonal anti-wheat antibody that was at room temperature for 1 h instead of 12 h at 8°C .

Conclusion

The ALERT gliadin screening test was more sensitive and faster than other tested methods. The indirect competitive ELISA developed in our laboratory was used as the reference method, but it was not sensitive enough with regard to the upper limit of the

acceptable amount of gluten in gluten-free food products as stated by Commission Regulation (EC) No. 41/2009 (European Commission 2009). An immunohistochemical procedure, eventually complemented by special staining of starch, seems to be a suitable diagnostic method and it was more sensitive than ELISA. However, it was time-consuming. Therefore, we shortened this procedure to 1 day only. In addition, three samples were sufficient for at least the detection of one wheat protein at a concentration of 1 g kg^{-1} (and greater) with a confidence level greater than 95%.

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