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

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

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Manuscripts

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3 **1 Comparison of immunohistochemical, histochemical and immunochemical**  
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6 **2 methods for detection of wheat protein allergens in meat samples and**  
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9 **3 cooked, dry, raw and fermented sausage samples**  
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1  
2  
3 22 **Abstract**  
4

5 23 Nowadays is it a common practice to add vegetable protein in the production of meat  
6  
7 24 products. Because of the possible substitution of high quality raw meat with vegetable protein  
8  
9 25 without the labeling the product package and because of the allergenic potential of many  
10  
11 26 vegetable proteins, it is important to develop as accurate methods for its detection. The  
12  
13 27 objective of the present study was to compare histochemical, immunochemical (ELISA,  
14  
15 28 ALERT gliadin screening test), and immunohistochemical methods for detection of wheat  
16  
17 29 protein in meat samples and sausages. Histochemical methods were useful for detection of  
18  
19 30 flour in meat samples, but immunohistochemical method was better for detection of wheat  
20  
21 31 protein. ALERT gliadin screening test detected gliadin from 10 mg kg<sup>-1</sup> while an  
22  
23 32 immunohistochemical method detected wheat protein concentrations from 1 g kg<sup>-1</sup> and  
24  
25 33 ELISA method detected wheat protein concentrations from 4 g kg<sup>-1</sup>. ALERT gliadin  
26  
27 34 screening test showed results within one day, whilst an ELISA detection method took two  
28  
29 35 days, and an immunohistochemical procedure took 5 days at the soonest, all including sample  
30  
31 36 preparation. This study also focused on optimization of an immunohistochemical method for  
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33 37 samples of cooked sausage. In addition, three samples were sufficient for wheat protein  
34  
35 38 detection at concentration of 1 g kg<sup>-1</sup> (and greater) with confidence level greater than 95 %.

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37 39  
38  
39 40 **Keywords:** Food allergens, wheat proteins, gluten, gliadin, histochemistry,  
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41 41 immunochemistry, immunohistochemistry  
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## 43 Introduction

44 During food production, various plant-origin proteins are used as additives in order to  
45 improve nutritional, flavour, and visual properties of food products (Pickering et al. 1995).  
46 However, plant proteins can be major allergens which can cause an atopic eczema (Sicherer  
47 and Sampson 1999), gastrointestinal disturbances, or other health complications in sensitive  
48 individuals (Hischenhuber et al. 2005). In production of foodstuffs of plant as well as animal  
49 origin, common allergens include wheat gluten containing gliadin and prolamin proteins.  
50 These are present also in wheat flour (Day et al. 2006) and are very important for dough,  
51 pastry and biscuits and pasta too. Gluten-containing proteins in incorrectly labeled products  
52 can have negative impact on the health of the consumers suffering from celiac disease, or  
53 gluten allergy (Ciclitira et al. 2005). The addition of plant proteins can negatively influence  
54 guaranteed storage period of some meat products, since it increases water binding capacity in  
55 foodstuffs (Vaňha et al. 2002). Plant proteins can also be used to substitute animal protein,  
56 which may cause, in case of inadequate product labeling, food adulteration (Pospiech et al.  
57 2009).

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

63 The development of reliable and fast methods for detection of plant-origin proteins,  
64 particularly of plant allergens, is essential (Poms et al. 2004). These requirements are met, for  
65 example, by these methods: immunochemical procedures (Moriyama et al. 2005)  
66 (radioimmunoanalysis, immunoelectrophoresis, enzymatic immunoanalytical methods –  
67 ELISA) (Asensio et al. 2008; Thompson and Méndez 2008), PCR – polymerase chain

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3 68 reaction (Zeltner et al. 2009), but also immunohistochemical methods (Pospiech et al. 2009).  
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5 69 Another possibility includes mass spectrometry (Šalplachta et al. 2005). Nevertheless, all  
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7  
8 70 these methods are not suitable for common food examination, in particular to meet the needs  
9  
10 71 of the food control authorities.

11  
12  
13 72 The detection of allergens in food products can be very difficult, as they are often  
14  
15 73 present only in trace amounts or are masked by the food matrix (Poms et al., 2004).  
16  
17 74 Immunological techniques are generally very sensitive and specific and have become very  
18  
19 75 popular for detection of small amounts of allergens. These methods based on the reactivity  
20  
21 76 between the antigen and the antiserum recognize only particular regions of the protein with a  
22  
23 77 characteristic structure called epitopes (Belloque et al., 2002). This mechanism is the same for  
24  
25 78 immunohistochemical methods which we used to develop histological and high-sensitive  
26  
27 79 immunological methods.

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30  
31 80 With regard to the fact that the amount of allergenic protein inducing allergic reaction  
32  
33 81 shifts within a broad range of concentrations and is specific for each individual (Stern et al.  
34  
35 82 2001), an important monitored aspect is the reliability of detection of added wheat protein.  
36  
37 83 The aim of this study was to compare histochemical, immunochemical, and  
38  
39 84 immunohistochemical methods for detection of wheat protein in meat and sausage samples  
40  
41 85 with and without additives.  
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## 48 87 **Materials and methods**

### 49 50 88 *Preparation of meat samples*

51  
52  
53 89 Meat samples were prepared from pork meat without additive (control) and with 10 g kg<sup>-1</sup>, 2 g  
54  
55 90 kg<sup>-1</sup>, 3 g kg<sup>-1</sup>, 4 g kg<sup>-1</sup>, 5 g kg<sup>-1</sup>, 10 g kg<sup>-1</sup>, 25 g kg<sup>-1</sup>, and 50 g kg<sup>-1</sup> of wheat protein (Amylon,  
56  
57 91 a. s., Havlíčkův Brod, Czech Republic). Other meat samples were prepared from pork meat  
58  
59 92 with 5 g kg<sup>-1</sup>, 10 g kg<sup>-1</sup>, 25 g kg<sup>-1</sup>, and 50 g kg<sup>-1</sup> of wheat medium flour (Penam, a. s., Brno,

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2  
3 93 Czech Republic). Another meat sample was prepared from pork meat with 10 g kg<sup>-1</sup> wheat  
4  
5 94 protein and 10 g kg<sup>-1</sup> wheat soft flour (Penam, a. s., Brno, Czech Republic).  
6  
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10 96 ***Preparation of sausage samples***  
11

12 97 Sausage samples were manufactured based on recipes described by Šedivý (1998). The  
13  
14 98 products included cooked sausage, raw sausage, dry sausage, and fermented sausage without  
15  
16 99 additive (control) and with 1 g kg<sup>-1</sup>, 5 g kg<sup>-1</sup>, 15 g kg<sup>-1</sup>, and 30 g kg<sup>-1</sup> of wheat protein  
17  
18 100 (Amylon, a. s., Havlíčkův Brod, Czech Republic). The wheat protein was added during  
19  
20 101 processing in a sausage cutter.  
21  
22  
23  
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27 103 ***Sample treatment and preparation***  
28

29 104 For ELISA method and ALERT gliadin screening test, samples in size of 250 g were taken  
30  
31 105 from each meat and sausage sample and subsequently frozen. Other samples in size of 1 cm<sup>3</sup>  
32  
33 106 were taken from meat samples. Samples in size of 1 cm<sup>3</sup> were taken from sausage samples of  
34  
35 107 cooked sausage from six locations (on either end and in the middle of the product always  
36  
37 108 from the center and subsurface layer). As the seventh sample of cooked sausage, homogenate  
38  
39 109 was prepared from either end and from the middle of the product by means of grinding in a  
40  
41 110 blender TM 4510 type FP 2111 (Concept, Hong Kong, China). The sample in size of 1 cm<sup>3</sup>  
42  
43 111 from raw sausage, dry sausage, and fermented sausage was only taken from the center of  
44  
45 112 model meat product.  
46  
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49

50 113 The samples were processed for histological and immunohistochemical examination  
51  
52 114 according to the procedure described by Pospiech et al. (2009). Samples (1 cm<sup>3</sup>) were fixed in  
53  
54 115 10% water solution of neutral formalin for more than 24 hours. After fixation, the samples  
55  
56 116 were dewatered in ascending sequence of alcohol in the autotechnicon apparatus Leica  
57  
58 117 TP1020 – Automatic Tissue Processor for Histology Applications (Leica Microsystems  
59  
60

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2  
3 118 GmbH, Wetzlar, Germany), and embedded into paraffin blocks in Paraplaste that were cut to  
4  
5 119 4 µm sections at rotation microtome Leica RM2255 (Leica Microsystems GmbH, Wetzlar,  
6  
7  
8 120 Germany). Model meat product samples of cooked sausage were cut into 75 sections (25  
9  
10 121 slides with three sections each), while always 50 µm were trimmed between individual  
11  
12 122 sections. The sections were spread on the water surface and mounted on slides SuperFrost  
13  
14  
15 123 plus (Menzel-Gläser, Menzel GmbH & Co KG, Braunschweig, Germany).  
16  
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18 124

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20 125 ***Immunohistochemical examination of samples***  
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22 126 In total 117 sections from 13 meat samples (nine sections from each sample) were processed  
23  
24 127 immunohistochemically. Further, 2625 sections of cooked sausage and six sections of every  
25  
26 128 other sausage samples were examined immunohistochemically.  
27  
28

29 129 The procedure for immunohistochemical detection of wheat protein based on avidin –  
30  
31 130 biotin complex (ABC), derived from procedure described by Pospiech et al. (2009) was used.  
32  
33 131 This indirect three-stage method is more amplification method which uses high binding  
34  
35 132 affinity between avidin and biotin for antibodies detection. At first a polyclonal anti-wheat  
36  
37 133 antibody isolated from a rabbit (Sigma-Aldrich Company, St. Louis, USA) in dilution 1:1000  
38  
39 134 diluted with antibody diluent (DakoCytomation ref. S0809, Glostrup, Denmark) is bound to  
40  
41 135 an antigen. In negative control, the primary antibody was substituted by antibody diluent  
42  
43 136 (DakoCytomation ref. S0809, Glostrup, Denmark). In the second phase, a biotin-conjugated  
44  
45 137 secondary antibody (VectorLaboratories, PK 6101, Burlingtone, USA) is attached to the  
46  
47 138 bound primary antibody. The third stage is bond between avidin – biotin complex –  
48  
49 139 peroxidase from the ABC reagent (VectorLaboratories, PK 6101, Burlingtone, USA) and  
50  
51 140 secondary biotinylated antibody which significantly amplifies signal.  
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57 141 The background was visualized in Calleja bath for 5 min or in toluidine bath for 1 min  
58  
59 142 and washed in water bath while some sections were left without background staining. Meat  
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3 143 samples containing  $10 \text{ g kg}^{-1}$  wheat protein as well as  $10 \text{ g kg}^{-1}$  wheat soft flour were after  
4  
5 144 immunohistochemical detection stained by a combination of PAS – Calleja (Protocols 1997),  
6  
7 145 Lugol – Calleja (Protocols 1989), Lugol (Bancroft at al. 1994) – toluidine blue (Flint 1994),  
8  
9  
10 146 and PAS – toluidine blue staining (Bancroft at al. 1994). Summary of all used examinations is  
11  
12 presented in Table 1.  
13

14  
15 148 PAS – Calleja staining – sections were immersed in: (1) orthoperiodic acid for 10 min,  
16  
17 149 (2) washed in reduction bath and then (3) in 70% aqueous ethanol (v/v), (4) Schiff's reagent  
18  
19 150 for 15 min, (5) washed in water bath for 15 min, (6) Calleja bath for 5 min and washed in  
20  
21 151 water bath.  
22  
23

24  
25 152 Lugol – Calleja staining – sections were immersed in: (1) Lugol bath for 5 min, (2)  
26  
27 153 Calleja bath for 5 min and washed in water bath.  
28

29 154 Lugol – toluidine blue staining – sections were immersed in: (1) Lugol bath for 5 min,  
30  
31 155 (2) toluidine bath for 1 min and washed in water bath.  
32  
33

34 156 PAS – toluidine blue staining: sections were immersed in: (1) orthoperiodic acid for  
35  
36 157 10 min, (2) washed in reduction bath and then (3) in 70% aqueous ethanol (v/v), (4) Schiff's  
37  
38 158 reagent for 15 min, (5) washed in water bath for 15 min, (6) toluidine bath for 1 min and  
39  
40 159 washed in water bath.  
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#### 45 46 161 *Histochemical examination of samples*

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48 162 From selected meat samples (meat with  $25 \text{ g kg}^{-1}$  addition of wheat protein, meat with  $25 \text{ g}$   
49  
50 163  $\text{kg}^{-1}$  addition of wheat flour), twelve sections were stained by general microscopic staining  
51  
52 164 (six sections hematoxylin – eosin (Bancroft at al. 1994), six sections toluidine blue (Flint  
53  
54 165 1994)), twelve sections by special microscopic staining (six sections Lugol – Calleja  
55  
56 166 (Protocols 1989), six sections PAS – Calleja (Protocols 1997)). Summary of all used  
57  
58 167 examinations is presented in Table 1.  
59  
60

168

169 *The examination procedure*

170 After histochemical and immunohistochemical detection, the total number of 2766 sections  
171 were examined in a light microscope Nikon ECLIPSE E200 (Fuji Bldg, Tokyo, Japan) at  
172 magnification of 40x. In case of obscurities, magnifications of 100x and 400x were used. A  
173 set of digital photographs was captured to document the results by means of a digital camera  
174 Canon PowerShot G9 (Canon inc., Japan) with use of picture capturing software PSRemote  
175 Version 1.5.2 (Breeze systems, Bagshot, UK).

176

177 *Immunochemical examination of samples*178 *ELISA method*

179 The indirect competitive ELISA was prepared according to Rencova et al. (2000) and  
180 Rencova and Tremlova (2009) and then it was modified for the detection of wheat gliadin in  
181 model meat samples. Meat samples with wheat protein were simultaneously tested for the  
182 presence of wheat proteins.

183 The immunization antigen was prepared from purified wheat gliadin (Sigma-Aldridge,  
184 USA). The amount of 5 mg of gliadin was dissolved in 1 ml of 70 % ethanol v/v with the  
185 addition of 10 µl of acetic acid. This volume was adjusted to 5 ml with the protein  
186 concentration of 1 mg ml<sup>-1</sup>. Three mice BALB/C of 10 weeks old and 22 g weight were  
187 immunized using purified gliadin antigen dissolved in 70% ethanol mixed 1:1 with complete  
188 Freund's adjuvant (Sigma Aldridge, USA) administered subcutaneously. The antigen  
189 concentration was 50 µg ml<sup>-1</sup> and the immunization dose was 0.1 ml. Boosters were applied  
190 twice in three weeks intervals 1:1 with Freund's incomplete adjuvant. Test sera were collected  
191 by tail bleeding 10 days after each injection; the titer of the sera was then determined by  
192 indirect ELISA. The last booster was applied intraperitoneally 4 days before the fusion.

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3 193 Spleen cells were fused with the myeloma cell line (P3x63.Ag8.653., ATCC CRL 1580) at a  
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5 194 ratio of 5:1 in the presence of polyethylene glycol. The next procedure was applied according  
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7  
8 195 to Chi-Chen et al. (2001). The purified antibodies were titrated against gliadin using indirect  
9  
10 196 ELISA.

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12 197 Meat samples of 20 g were processed in the blender with 200 ml of 70% ethanol v/v.  
13  
14 198 After a 30 min extraction process, the extract solution was centrifuged at 10 000 x g and 4°C  
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16 199 for 10 min and the supernatant was used for analysis.  
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#### 22 201 *ALERT gliadin screening test*

23  
24 202 For comparison of results achieved by the above described procedures, sausage samples were  
25  
26 203 examined also by ALERT gliadin screening test (Neogen corporation, Lansing, USA)  
27  
28 204 designed for qualitative analysis of gluten-free food products for the presence of gliadins and  
29  
30 205 prolamins found in wheat, barley and rye. The producer claims that the test is able to detect  
31  
32 206 already addition of 10 mg kg<sup>-1</sup> of these allergens. ALERT test is based on principle of  
33  
34 207 sandwich enzyme-linked immunosorbent assay. Gliadin is first extracted from samples with a  
35  
36 208 40% dilution of ethanol. Extract is diluted in Phosphate Buffered Saline (PBS), 80g l<sup>-1</sup> NaCl,  
37  
38 209 2 g l<sup>-1</sup> KCl, 2 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 23,4 g l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>\*12H<sub>2</sub>O, 0,16 g l<sup>-1</sup> NaOH adjusted to pH 7,4,  
39  
40 210 and applied to antibody-coated wells where gluten will bind to the antibody during an  
41  
42 211 incubation period. Any unbound gliadin is washed away and second antibody, which is  
43  
44 212 enzyme labeled, is added. The detector antibody binds to the gliadin during another  
45  
46 213 incubation period. Unbound antibody is washed away and a one step substrate is added. Color  
47  
48 214 develops as a result of the presence of bound-labeled antibody. Blue color indicates samples  
49  
50 215 containing high levels of gliadin while purple or red samples contain little or no gliadin. Color  
51  
52 216 is compared to negative and positive control.  
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3 218 *Statistical analysis*  
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5 219 Statistical evaluation was performed by statistical software Minitab 15 (Quality Plaza, State  
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7  
8 220 College PA, USA). We used logistic regression analysis to find out how many sections from  
9  
10 221 the sausage samples are necessary to examine to be able to decide with the confidence of  
11  
12 222 95 % that the wheat protein is present in the product.  
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14

15 223

## 17 224 **Results and discussion**

18  
19 225 Special staining (Lugol – Calleja and PAS – Calleja staining) indicated the presence of flour  
20  
21 226 on the basis of different colors on the microscope slide. Lugol – Calleja staining indicated the  
22  
23 227 presence of flour starch particles in meat samples that were within dark purple and black  
24  
25 228 (Figure 1) and PAS – Calleja staining highlighted all polysaccharides in pink. The staining  
26  
27 229 results for meat samples were the same at different spiking levels of wheat flour. Other  
28  
29 230 components of meat samples were evident as various tones of green. On the other hand,  
30  
31 231 general staining (hematoxylin – eosin, toluidine blue) indicated flour only on the basis of their  
32  
33 232 typical appearance and structure in microscopic picture (Figure 2). Flour formed clusters  
34  
35 233 containing particles of wheat starch that looked similar to small and big lentil-like kernels. A  
36  
37 234 similar finding was reported by Tremlová and Štarha (2002). However, the shape of wheat  
38  
39 235 starch can be changed after technological processing (Aguilera and Stanley 1990).  
40  
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44

45 236 Wheat protein in meat samples was histochemically identified only on the basis of  
46  
47 237 their typical spongy structure with openings (Figure 3). Moreover, special staining of starch  
48  
49 238 indicated sporadic starch granules contained in wheat protein. In contrast to flour, wheat  
50  
51 239 protein was not significantly differentiated based on different color from other components.  
52  
53 240 Only general staining with toluidine blue gave plant-origin proteins colours in different  
54  
55 241 shades of blue (wheat protein is light cyan) from other product components (Flint 1994).  
56  
57 242 Simultaneously, wheat protein in meat samples was detected by immunohistochemical  
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3 243 methods where wheat protein was visualized as brown particles by DAB (3,3'-  
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5 244 Diaminobenzidine) chromogene (Figure 4) in contrast to stained background with toluidine  
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8 245 blue (blue) or with Calleja staining (green). The staining results for meat samples and sausage  
9  
10 246 samples were the same at different spiking levels of wheat protein. However, this method had  
11  
12 247 limits in detection of wheat flour, where this staining caused only minor changes (Figure 5).  
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14  
15 248 This phenomenon was probably caused by relatively low concentration of wheat protein in  
16  
17 249 wheat flour (70 – 130 g kg<sup>-1</sup> proteins) (Keletunç and Breslauer 2003), so individual protein  
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19 250 epitopes with conjugated antibodies were too far away from each other and did not present a  
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21  
22 251 colorful picture visible to one's eye.

23  
24  
25 252 Based on the above described results, meat samples containing wheat protein as well  
26  
27 253 as wheat flour were subjected to the combination of immunohistochemical and histochemical  
28  
29 254 examination specialized on starch detection. Various methods for preparation staining were  
30  
31 255 used to stain backgrounds after immunohistochemical processing. Combinations used were  
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34 256 PAS – Calleja, Lugol – Calleja, and, for stronger contrast between wheat protein highlighted  
35  
36 257 by DAB chromogene and other components, also Lugol – toluidine blue and PAS – toluidine  
37  
38 258 blue. From the above combinations, PAS – Calleja staining (Figure 6) provided the largest  
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40  
41 259 color differences between the monitored components. Combinations with Lugol solution  
42  
43 260 staining resulted in weaker color contrast between starch and wheat protein and partially in  
44  
45  
46 261 interference of individual stains.

47  
48 262 Immunohistochemical detection of meat samples was compared to results achieved by  
49  
50 263 reference ELISA method. The ELISA method reliably detected wheat protein concentrations  
51  
52 264 from 4 g kg<sup>-1</sup> while immunohistochemical method detected wheat protein concentrations from  
53  
54  
55 265 1 g kg<sup>-1</sup> (Table 2). Generally acknowledged requirement on detection limit for allergen is a  
56  
57 266 value of 1 to 100 mg kg<sup>-1</sup> of allergens in food products, depending on allergen type, possibly  
58  
59  
60 267 depending on analytical possibilities (Koppelman and Hefle 2006). Upper limit of this range

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3 268 is identical with upper limit of acceptable amount of gluten in gluten-free food products as  
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5 269 stated by Commission Regulation (EC) No. 41/2009 concerning composition and labeling of  
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8 270 foodstuffs suitable for people intolerant to gluten. It establishes that retail-sale form of food  
9  
10 271 products for people intolerant to gluten must not contain more than 20 mg kg<sup>-1</sup> of gluten in  
11  
12 272 case they do not include any constituents from wheat or other types of *Triticum*, or more than  
13  
14  
15 273 100 mg kg<sup>-1</sup> of gluten in case constituents from wheat or other types of *Triticum* were used at  
16  
17 274 their production.

18  
19  
20 275 Detection of wheat protein by ELISA method, commercially available ALERT gliadin  
21  
22 276 screening test, and immunohistochemical procedure with toluidine blue staining in sausage  
23  
24 277 samples is shown in Table 3. The only method with negative detection of samples with wheat  
25  
26 278 protein was the ELISA method (Table 2 and 3). We suppose that this negative detection was  
27  
28 279 possibly caused by decreased sensitivity of detection method (detection limit 4 – 5 g kg<sup>-1</sup>), or  
29  
30 280 possibly, in cooked products, by worsened availability of antigen after technological  
31  
32 281 processing of the product. The only method which met the commonly acknowledged  
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34 282 detection limit was the ALERT gliadin screening test. Producer of this test claims ability to  
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36 283 detect gliadin already from 10 mg kg<sup>-1</sup> (20 mg kg<sup>-1</sup> of gluten) in this test, which is confirmed  
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38 284 also by results of this study. However, in our control sausage samples of dry sausage and raw  
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40 285 sausage without any wheat protein addition this test showed false positive reaction. This  
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42 286 might have been caused by cross-contamination of samples during preparation for  
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44 287 examination, which included sample grinding (in one machine). Including sample  
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46 288 preparation, immunohistochemical procedure showed results in 5 days at the soonest, while  
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48 289 ELISA detection method in two days, and ALERT gliadin test within one day.

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51 290 Immunohistochemical method seems to be sensitive and reliable method that contains  
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53 291 current possibilities of food microscopy – detection of food components, quality detection,  
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55 292 and arrangement of individual food components, adulteration of foodstuffs, risk components

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3 293 detection in food products, and possible quantification of food components (Heertje and  
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5 294 Leunis 1997). Microscopy represents an effective and in many cases irreplaceable instrument  
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8 295 for achieving necessary information. Unlike immunochemical methods that can mostly detect  
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10 296 only one constituent in one detection, food microscopy allows for detection of several food  
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12 297 components within one analysis (Heertje et al. 1997).

15 298 Our study was also focused on optimization of immunohistochemical procedure,  
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17 299 which was performed in sausage samples of cooked sausage. We found out how many  
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19 300 sections from the sausage sample are necessary to examine to be able to decide with the  
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21 301 confidence of 95 % that the wheat protein is present in the product. If the test of dependence  
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23 302 of wheat protein concentration with respect to the probability of detection (using logistic  
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25 303 regression) is made, wheat protein concentration will be significant at significance level of  
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27 304 0.05. If the information, that there are 7 types of samples, is added into model, also the factor  
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29 305 “sample type” will be significant at significance level of 0.05. It is a problem because we  
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31 306 would make a model where the wheat protein detection is examined with respect to the  
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33 307 concentration and sample type. But we do not have enough data for this model. We focused  
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35 308 on the sample with the worst detection ratio which was the homogenate of cooked sausage  
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37 309 with 1 g kg<sup>-1</sup> addition of wheat protein (Table 4). The point estimate for probability of wheat  
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39 310 protein detection was  $63/75 = 0.84$ . If only one section was made, the probability of wheat  
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41 311 protein detection was  $p = 0.753692$  using one-sided interval estimate with confidence 95%. If  
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43 312 two sections were made, the probability of wheat protein detection was  $1 - (1 - p)^2 = 0.9393$ ,  
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45 313 which was still less than 0.95. If three sections were made, the probability of wheat protein  
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47 314 detection was  $1 - (1 - p)^3 = 0.9850$ , which was greater than 0.95. Therefore, three sections  
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49 315 were sufficient for at least one wheat protein detection at concentration of 1 g kg<sup>-1</sup> (and  
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51 316 greater) with confidence level greater than 95 %. The calculated minimum number of  
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53 317 examined sections necessary for reliable detection then needs to be increased by losses caused  
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3 318 by harms to sections during processing (e.g. section drifting away while staining), which were  
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5 319 6.1 %. The optimization also included shortening the time of immunohistochemical method.  
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8 320 In order to decrease time consumption, this procedure was shorted to one day. The main  
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10 321 change was especially in incubation with an polyclonal anti-wheat antibody that was for 1  
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12 322 hour at room temperature instead of 12 h at 8°C.  
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323

### 324 **Conclusion**

325 ALERT gliadin screening test was more sensitive and faster than other tested methods.  
326 Indirect competitive ELISA developed in our laboratory was used as the reference method,  
327 but it was not sensitive enough with regard to upper limit of acceptable amount of gluten in  
328 gluten-free food products as stated by Commission Regulation (EC) No. 41/2009. A  
329 immunohistochemical procedure, eventually complemented by special staining of starch,  
330 seems to be a suitable diagnostic method and it was more sensitive than ELISA, however, it  
331 was time-consuming. Therefore, we shortened this procedure to one day only. In addition,  
332 three samples were sufficient for at least the detection of one wheat protein at a concentration  
333 of 1 g kg<sup>-1</sup> (and greater) with confidence level greater than 95 %.

334

### 335 **Acknowledgements**

336 This work was supported by project NAZV No. 1B53004, research intention of Veterinary  
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338 No. 206/2009/VFU.

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

Wheat protein additive	Sample	Number of sections with positive result	Number of sections with negative result	Number of examined sections	Losses during processing
1 g kg <sup>-1</sup>	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
	ZO	74	0	74	1
5 g kg <sup>-1</sup>	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
15 g kg <sup>-1</sup>	HO	72	3	75	0
	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	

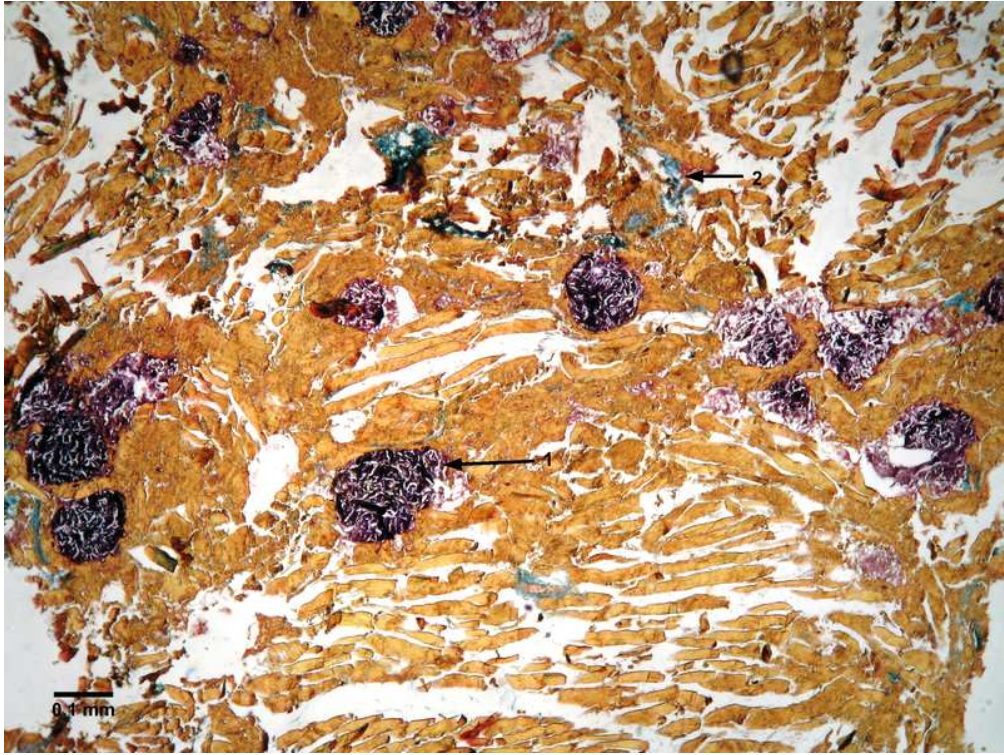
	KS	71	0	71	4
	HO	72	0	72	3
	ZO	75	0	75	0
	PO	75	0	75	0
	KO	75	0	75	0
30 g kg <sup>-1</sup>	ZS	75	0	75	0
	PS	75	0	75	0
	KS	75	0	75	0
	HO	73	1	74	1
	ZO	0	37	37	38
	PO	0	70	70	5
	KO	0	48	48	27
Control	ZS	0	63	63	12
	PS	0	65	65	10
	KS	0	60	60	15
	HO	0	68	68	7
Sum		2010	454	2464	161

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- 3 ZO ..... product beginning, undercover layer
  - 4 PO ..... product middle part, undercover layer
  - 5 KO ..... product end, undercover layer
  - 6 ZS ..... product beginning, product center
  - 7 PS ..... product middle part, product center
  - 8 KS ..... product end, product center
  - 9 HO ..... homogenate from various parts of the product

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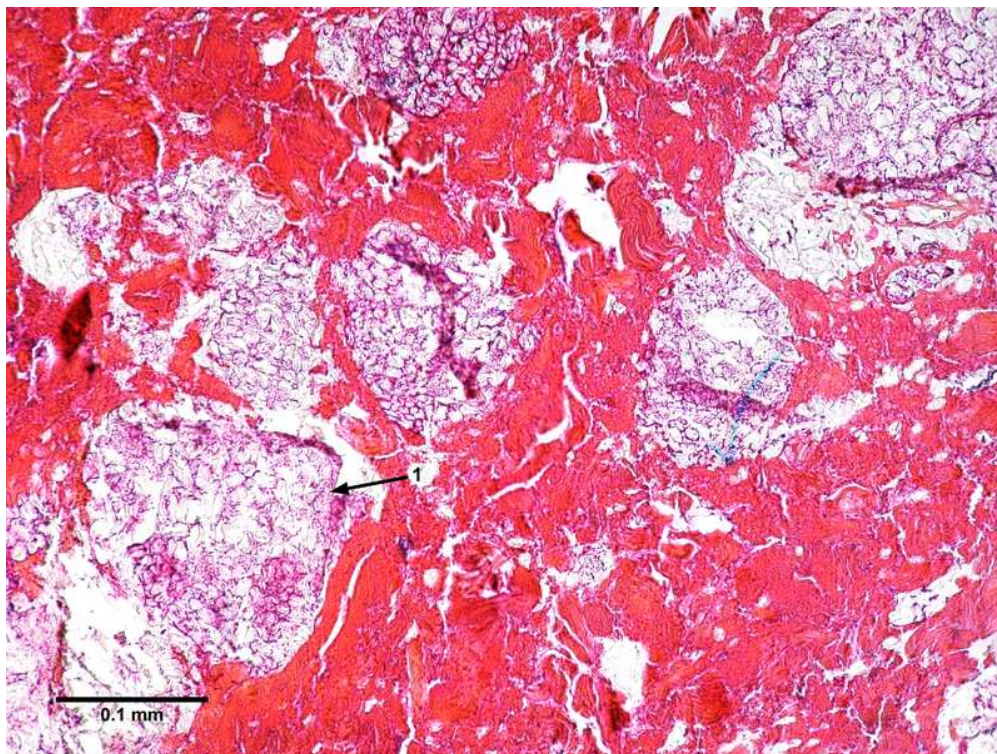
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Meat sample – meat with wheat flour, Lugol – Calleja staining, examined at 40x, (1) wheat flour, (2) collagen.  
67x50mm (300 x 300 DPI)



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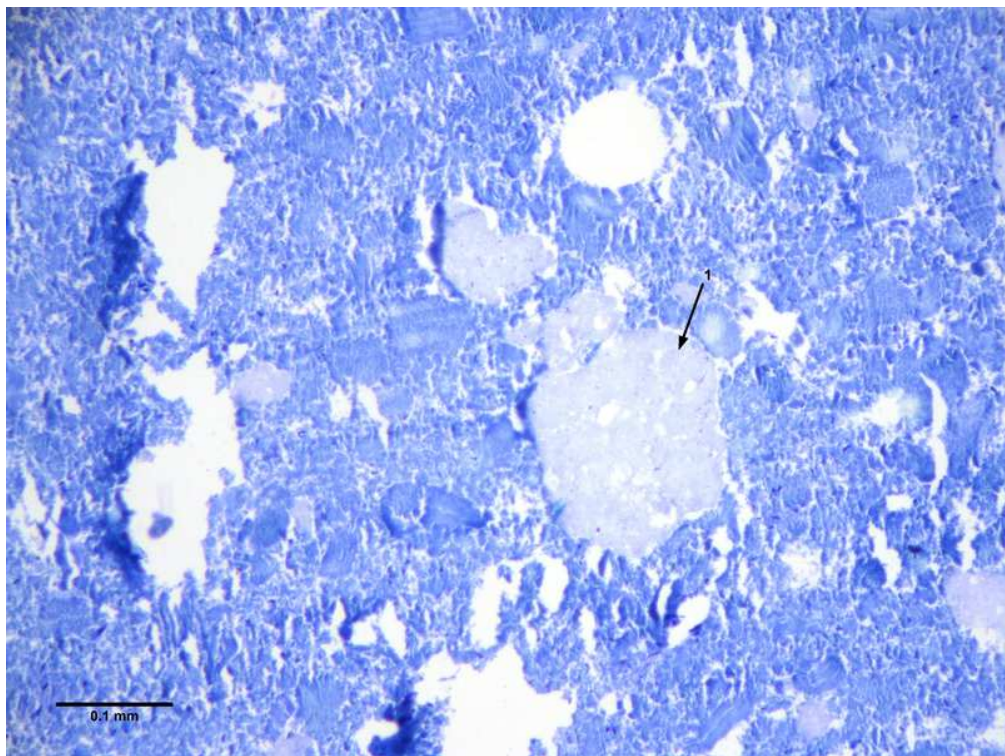


Meat sample – meat with wheat flour, Hematoxylin – Eosin staining, examined at 100x, (1) wheat flour.  
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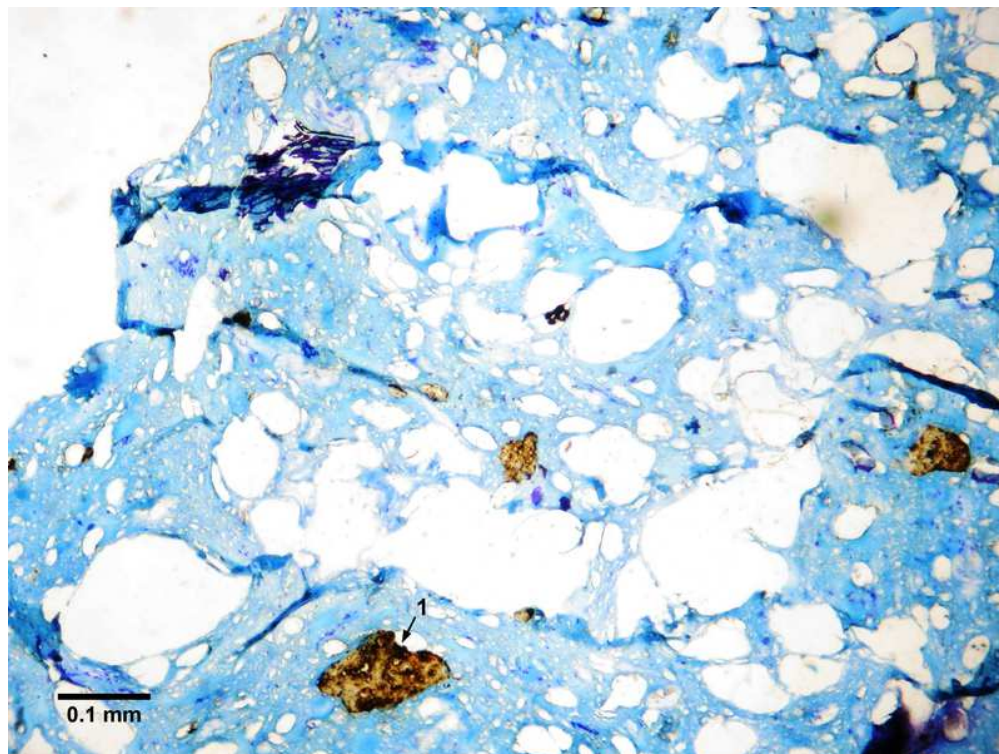


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Meat sample – meat with wheat protein, toluidine blue staining, examined at 100x, (1) wheat protein.  
67x50mm (300 x 300 DPI)

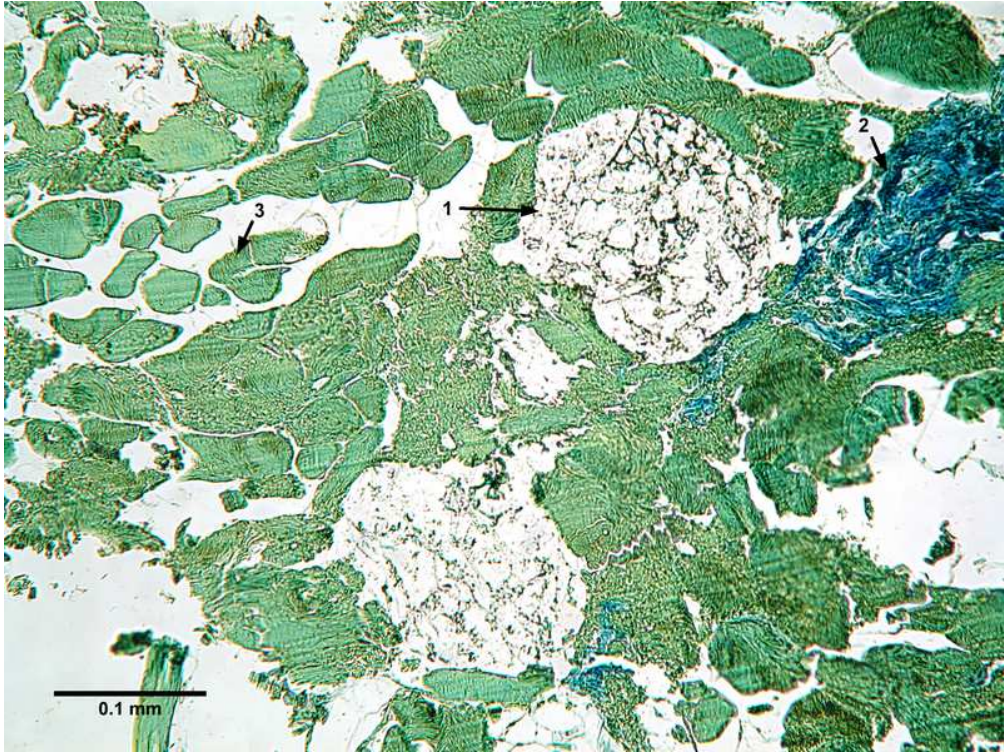
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Sausage sample – cooked sausage with wheat protein, immunohistochemical method, highlighted by DAB chromogene, toluidine blue staining, examined at 40x, (1) wheat protein.  
67x50mm (300 x 300 DPI)



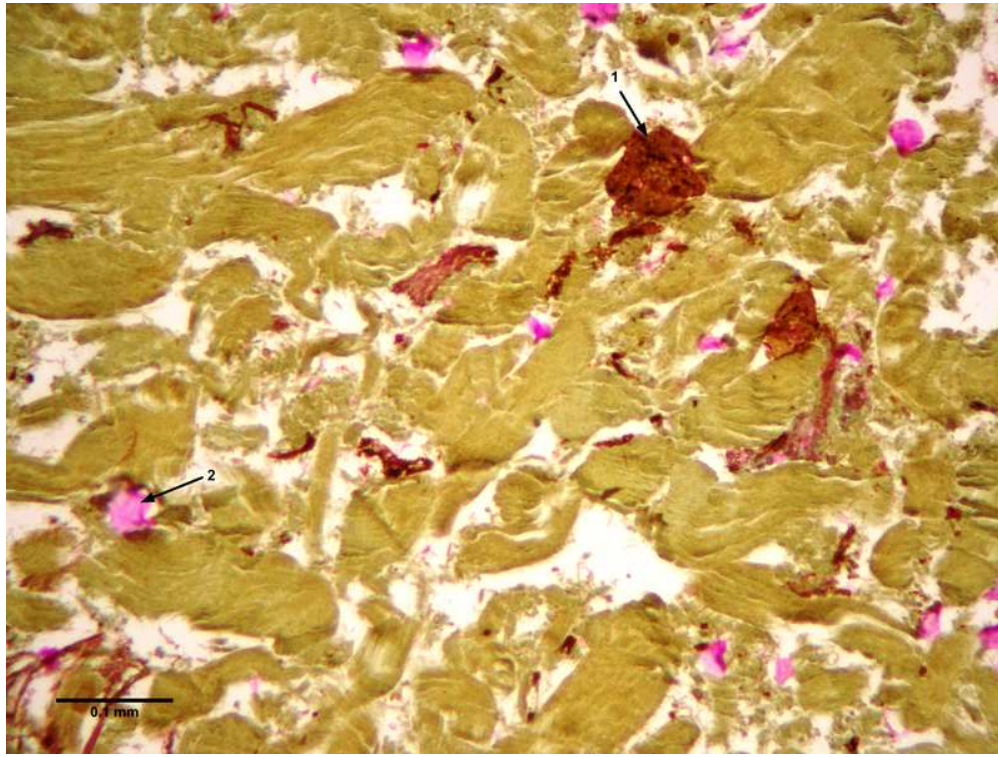
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Meat sample – meat with wheat flour, immunohistochemical method, highlighted by DAB chromogene, staining according to Calleja, examined at 100x, (1) flour, (2) collagen, (3) muscle tissue.

67x50mm (300 x 300 DPI)

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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.  
67x50mm (300 x 300 DPI)

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

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

+ ..... preparation background staining

- ..... examination not performed

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

Wheat protein addition [g kg <sup>-1</sup> ]	ELISA	Immunohistochemical method
0	-	-
1	-	+
2	+/-	+
3	+/-	+
4	+	+
5	+	+
10	+	+
25	+	+
50	+	+

+ ..... positive  
 - ..... negative  
 +/-.....dubious

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

Product	Wheat protein addition [g kg <sup>-1</sup> ]	ELISA	ALERT gliadin screening test	Immunohistochemical method
	0	-	-	-
	1	-	+	+
Cooked sausage	5	+	+	+
	15	+	+	+
	30	+	+	+
	0	-	+	-
	1	-	+	+
Dry sausage	5	+	+	+
	15	+	+	+
	30	+	+	+
	0	-	+	-
	1	+	+	+
Raw sausage	5	+	+	+
	15	+	+	+
	30	+	+	+
	0	+	-	-
	1	+	+	+
Fermented sausage	5	+	+	+
	15	+	+	+
	30	+	+	+

+ ..... positive

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- ..... negative

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3 1 Table 4. Summary of immunohistochemical examination of sausage samples of cooked  
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Wheat protein additive	Sample	Number of sections with positive result	Number of sections with negative result	Number of sections examined	Losses during processing
1 g kg <sup>-1</sup>	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
	ZO	74	0	74	1
5 g kg <sup>-1</sup>	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
15 g kg <sup>-1</sup>	HO	72	3	75	0
	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
	ZO	75	0	75	0
	PO	75	0	75	0
	KO	75	0	75	0
30 g kg <sup>-1</sup>	ZS	75	0	75	0
	PS	75	0	75	0
	KS	75	0	75	0
	HO	73	1	74	1
	ZO	0	37	37	38
	PO	0	70	70	5
	KO	0	48	48	27
Control	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

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- 3 ZO ..... product beginning, undercover layer
- 4 PO ..... product middle part, undercover layer
- 5 KO ..... product end, undercover layer
- 6 ZS ..... product beginning, product center
- 7 PS ..... product middle part, product center
- 8 KS ..... product end, product center
- 9 HO ..... homogenate from various parts of the product