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

Z. Řezáčová Lukášková ^{1*}, B. Tremlová ¹, M. Pospiech ¹, E. Renčová ², Z. Randulová ¹, L. Steinhauser ³, A. Reichová ¹, J. Bednář ⁴

¹ Department of Vegetable Foodstuff and Plant Production, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1-3, 61242 Brno, Czech Republic

² Veterinary Research Institute, Brno, Czech Republic

³ Department of Meat Hygiene and Technology, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic

⁴ Department of Statistics and Optimization, Institute of Mathematics Faculty of Mechanical Engineering, Brno University of Technology, Czech Republic

*Correspondence: Z. Řezáčová Lukášková. E-mail: H07310@VFU.CZ

Abstract

Nowadays is it 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 labeling the product package and because of the allergenic potential of many vegetable proteins, it is important to develop as accurate methods for its detection. The objective of the present study was to compare histochemical, immunochemical (ELISA, ALERT gliadin screening test), and immunohistochemical methods for detection of wheat protein in meat samples and sausages. Histochemical methods were useful for detection of flour in meat samples, but immunohistochemical method was better for 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 ELISA method detected wheat protein concentrations from 4 g kg^{-1} . ALERT gliadin screening test showed results within one day, whilst an ELISA detection method took two days, and an immunohistochemical procedure took 5 days at the soonest, all including sample preparation. This study also focused on optimization of an immunohistochemical method for samples of cooked sausage. In addition, three samples were sufficient for wheat protein detection at concentration of 1 g kg^{-1} (and greater) with confidence level greater than 95 %.

Keywords: Food allergens, wheat proteins, gluten, gliadin, histochemistry, immunochemistry, immunohistochemistry

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Introduction

During food production, various plant-origin proteins are used as additives in order to improve nutritional, flavour, and visual properties of food products (Pickering et al. 1995). However, plant proteins can be major allergens which can cause an atopic eczema (Sicherer and Sampson 1999), gastrointestinal disturbances, or other health complications in sensitive individuals (Hischenhuber et al. 2005). In 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 and biscuits and pasta too. Gluten-containing proteins in incorrectly labeled products can have negative impact on the health of the consumers suffering from celiac disease, or gluten allergy (Ciclitira et al. 2005). The addition of plant proteins can negatively influence guaranteed storage period of some meat products, since it increases water binding capacity in foodstuffs (Vaňha et al. 2002). Plant proteins can also be used to substitute animal protein, which may cause, in case of inadequate product labeling, food adulteration (Pospiech et al. 2009).

Wheat protein (gluten), wheat flour or breadcrumb, which also contained 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. The vegetarian foodstuffs production can contain also wheat germ. In addition, wheat protein can be contained in baking powder or another thickening agents.

The development of reliable and fast methods for 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, enzymatic immunoanalytical methods – ELISA) (Asensio et al. 2008; Thompson and Méndez 2008), PCR – polymerase chain

reaction (Zeltner et al. 2009), but 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 detection of small amounts of allergens. These methods based on the reactivity between the antigen and the antiserum recognize only particular regions of the protein with a characteristic structure called epitopes (Belloque et al., 2002). This mechanism is the same for immunohistochemical methods which we used to develop histological and high-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 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 10 g kg⁻¹, 2 g kg⁻¹, 3 g kg⁻¹, 4 g kg⁻¹, 5 g kg⁻¹, 10 g kg⁻¹, 25 g kg⁻¹, 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 g kg⁻¹, 10 g kg⁻¹, 25 g kg⁻¹, and 50 g kg⁻¹ of wheat medium flour (Penam, a. s., Brno,

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3 93 Czech Republic). Another meat sample was prepared from pork meat with 10 g kg⁻¹ wheat
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5 94 protein and 10 g kg⁻¹ wheat soft flour (Penam, a. s., Brno, Czech Republic).
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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
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14 98 products included cooked sausage, raw sausage, dry sausage, and fermented sausage without
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16 99 additive (control) and with 1 g kg⁻¹, 5 g kg⁻¹, 15 g kg⁻¹, and 30 g kg⁻¹ of wheat protein
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18 100 (Amylon, a. s., Havlíčkův Brod, Czech Republic). The wheat protein was added during
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20 101 processing in a sausage cutter.
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27 103 *Sample treatment and preparation*

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29 104 For ELISA method and ALERT gliadin screening test, samples in size of 250 g were taken
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31 105 from each meat and sausage sample and subsequently frozen. Other samples in size of 1 cm³
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33 106 were taken from meat samples. Samples in size of 1 cm³ were taken from sausage samples of
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35 107 cooked sausage from six locations (on either end and in the middle of the product always
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37 108 from the center and subsurface layer). As the seventh sample of cooked sausage, homogenate
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39 109 was prepared from either end and from the middle of the product by means of grinding in a
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41 110 blender TM 4510 type FP 2111 (Concept, Hong Kong, China). The sample in size of 1 cm³
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43 111 from raw sausage, dry sausage, and fermented sausage was only taken from the center of
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45 112 model meat product.
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50 113 The samples were processed for histological and immunohistochemical examination
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52 114 according to the procedure described by Pospiech et al. (2009). Samples (1 cm³) were fixed in
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54 115 10% water solution of neutral formalin for more than 24 hours. After fixation, the samples
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56 116 were dewatered in ascending sequence of alcohol in the autotechnicon apparatus Leica
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58 117 TP1020 – Automatic Tissue Processor for Histology Applications (Leica Microsystems
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GmbH, Wetzlar, Germany), and embedded into paraffin blocks in Paraplaste that were cut to 4 μm sections at rotation microtome Leica RM2255 (Leica Microsystems GmbH, Wetzlar, Germany). Model meat product samples of cooked sausage were cut into 75 sections (25 slides with three sections each), while always 50 μm were trimmed between individual sections. The sections were spread on the water surface 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 immunohistochemical detection of wheat protein based on avidin – biotin complex (ABC), derived from procedure described by Pospiech et al. (2009) was used. This indirect three-stage method is more amplification method which uses high binding affinity between avidin and biotin for antibodies detection. At first a polyclonal anti-wheat antibody isolated from a rabbit (Sigma-Aldrich Company, St. Louis, USA) in dilution 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, Glostrup, Denmark). In the second phase, a biotin-conjugated secondary antibody (VectorLaboratories, PK 6101, Burlingtone, USA) is attached to the bound primary antibody. The third stage is bond between avidin – biotin complex – peroxidase from the ABC reagent (VectorLaboratories, PK 6101, Burlingtone, USA) and secondary biotinylated antibody which significantly amplifies signal.

The background was visualized in Calleja bath for 5 min or in toluidine bath for 1 min and washed in water bath while some sections were left without background staining. Meat

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3 143 samples containing 10 g kg⁻¹ wheat protein as well as 10 g kg⁻¹ wheat soft flour were after
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5 144 immunohistochemical detection stained by a combination of PAS – Calleja (Protocols 1997),
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7 145 Lugol – Calleja (Protocols 1989), Lugol (Bancroft at al. 1994) – toluidine blue (Flint 1994),
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10 146 and PAS – toluidine blue staining (Bancroft at al. 1994). Summary of all used examinations is
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12 147 presented in Table 1.

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

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

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

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34 156 PAS – toluidine blue staining: sections were immersed in: (1) orthoperiodic acid for
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36 157 10 min, (2) washed in reduction bath and then (3) in 70% aqueous ethanol (v/v), (4) Schiff’s
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38 158 reagent for 15 min, (5) washed in water bath for 15 min, (6) toluidine bath for 1 min and
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40 159 washed in water bath.

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46 161 ***Histochemical examination of samples***

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

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 μ l of acetic acid. This volume was adjusted to 5 ml with the protein
186 concentration of 1 mg ml⁻¹. 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 μ g ml⁻¹ 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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8 195 to Chi-Chen et al. (2001). The purified antibodies were titrated against gliadin using indirect
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10 196 ELISA.
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13 197 Meat samples of 20 g were processed in the blender with 200 ml of 70% ethanol v/v.
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15 198 After a 30 min extraction process, the extract solution was centrifuged at 10 000 x g and 4°C
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17 199 for 10 min and the supernatant was used for analysis.
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22 201 *ALERT gliadin screening test*
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24 202 For comparison of results achieved by the above described procedures, sausage samples were
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26 203 examined also by ALERT gliadin screening test (Neogen corporation, Lansing, USA)
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28 204 designed for qualitative analysis of gluten-free food products for the presence of gliadins and
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30 205 prolamins found in wheat, barley and rye. The producer claims that the test is able to detect
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32 206 already addition of 10 mg kg⁻¹ of these allergens. ALERT test is based on principle of
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34 207 sandwich enzyme-linked immunosorbent assay. Gliadin is first extracted from samples with a
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36 208 40% dilution of ethanol. Extract is diluted in Phosphate Buffered Saline (PBS), 80g l⁻¹ NaCl,
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38 209 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,
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40 210 and applied to antibody-coated wells where gluten will bind to the antibody during an
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42 211 incubation period. Any unbound gliadin is washed away and second antibody, which is
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44 212 enzyme labeled, is added. The detector antibody binds to the gliadin during another
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46 213 incubation period. Unbound antibody is washed away and a one step substrate is added. Color
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48 214 develops as a result of the presence of bound-labeled antibody. Blue color indicates samples
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50 215 containing high levels of gliadin while purple or red samples contain little or no gliadin. Color
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52 216 is compared to negative and positive control.
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218 *Statistical analysis*

219 Statistical evaluation was performed by statistical software Minitab 15 (Quality Plaza, State
220 College PA, USA). We used logistic regression analysis to find out how many sections from
221 the sausage samples are necessary to examine to be able to decide with the confidence of
222 95 % that the wheat protein is present in the product.

224 **Results and discussion**

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

236 Wheat protein in meat samples was histochemically identified only on the basis of
237 their typical spongy structure with openings (Figure 3). Moreover, special staining of starch
238 indicated sporadic starch granules contained in wheat protein. In contrast to flour, wheat
239 protein was not significantly differentiated based on different color from other components.
240 Only general staining with toluidine blue gave plant-origin proteins colours in different
241 shades of blue (wheat protein is light cyan) from other product components (Flint 1994).
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
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10 246 samples were the same at different spiking levels of wheat protein. However, this method had
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12 247 limits in detection of wheat flour, where this staining caused only minor changes (Figure 5).
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15 248 This phenomenon was probably caused by relatively low concentration of wheat protein in
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17 249 wheat flour (70 – 130 g kg⁻¹ 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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22 251 colorful picture visible to one's eye.

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24 252 Based on the above described results, meat samples containing wheat protein as well
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27 253 as wheat flour were subjected to the combination of immunohistochemical and histochemical
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29 254 examination specialized on starch detection. Various methods for preparation staining were
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32 255 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
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36 257 by DAB chromogene and other components, also Lugol – toluidine blue and PAS – toluidine
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39 258 blue. From the above combinations, PAS – Calleja staining (Figure 6) provided the largest
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41 259 color differences between the monitored components. Combinations with Lugol solution
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43 260 staining resulted in weaker color contrast between starch and wheat protein and partially in
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46 261 interference of individual stains.

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

is identical with upper limit of acceptable amount of gluten in gluten-free food products as stated by Commission Regulation (EC) No. 41/2009 concerning composition and labeling of foodstuffs suitable for people intolerant to gluten. It establishes that 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.

Detection of wheat protein by ELISA method, commercially available ALERT gliadin screening test, and 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 2 and 3). We suppose that this negative detection was possibly caused by decreased sensitivity of detection method (detection limit 4 – 5 g kg⁻¹), or possibly, in cooked products, by worsened availability of antigen after technological processing of the product. The only method which met the commonly acknowledged detection limit was the ALERT gliadin screening test. Producer of this test claims ability to detect gliadin already from 10 mg kg⁻¹ (20 mg kg⁻¹ of gluten) in this test, which is confirmed also by results of this study. However, in our control sausage samples of dry sausage and raw sausage without any wheat protein addition this test showed 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, immunohistochemical procedure showed results in 5 days at the soonest, while ELISA detection method in two days, and ALERT gliadin test within one day.

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

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

298 Our study was also focused on optimization of immunohistochemical procedure,
299 which was performed in sausage samples of cooked sausage. We found out how many
300 sections from the sausage sample are necessary to examine to be able to decide with the
301 confidence of 95 % that the wheat protein is present in the product. If the test of dependence
302 of wheat protein concentration with respect to the probability of detection (using logistic
303 regression) is made, wheat protein concentration will be significant at significance level of
304 0.05. If the information, that there are 7 types of samples, is added into model, also the factor
305 "sample type" will be significant at significance level of 0.05. It is a problem because we
306 would make a model where the wheat protein detection is examined with respect to the
307 concentration and sample type. But we do not have enough data for this model. We focused
308 on the sample with the worst detection ratio which was the homogenate of cooked sausage
309 with 1 g kg^{-1} addition of wheat protein (Table 4). The point estimate for probability of wheat
310 protein detection was $63/75 = 0.84$. If only one section was made, the probability of wheat
311 protein detection was $p = 0.753692$ using one-sided interval estimate with confidence 95%. If
312 two sections were made, the probability of wheat protein detection was $1 - (1 - p)^2 = 0.9393$,
313 which was still less than 0.95. If three sections were made, the probability of wheat protein
314 detection was $1 - (1 - p)^3 = 0.9850$, which was greater than 0.95. Therefore, three sections
315 were sufficient for at least one wheat protein detection at concentration of 1 g kg^{-1} (and
316 greater) with confidence level greater than 95 %. The calculated minimum number of
317 examined sections necessary for reliable detection then needs to be increased by losses caused

by harms to sections during processing (e.g. section drifting away while staining), which were 6.1 %. The optimization also included shortening the time of immunohistochemical method. In order to decrease time consumption, this procedure was shorted to one day. The main change was especially in incubation with an polyclonal anti-wheat antibody that was for 1 hour at room temperature instead of 12 h at 8°C.

Conclusion

ALERT gliadin screening test was more sensitive and faster than other tested methods. Indirect competitive ELISA developed in our laboratory was used as the reference method, but it was not sensitive enough with regard to upper limit of acceptable amount of gluten in gluten-free food products as stated by Commission Regulation (EC) No. 41/2009. A 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 one 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 confidence level greater than 95 %.

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- 414 “Table 1”
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Table 4. Summary of immunohistochemical examination of sausage samples of cooked 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 ⁻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
	ZO	74	0	74	1
5 g kg ⁻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
	ZO	75	0	75	0
	PO	75	0	75	0
	KO	75	0	75	0
30 g kg ⁻¹	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

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

Meat sample – meat with wheat flour, Hematoxylin – Eosin staining, examined at 100x, (1) wheat flour.
67x50mm (300 x 300 DPI)

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

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)

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)

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	ELISA	+ Calleja
	(hematoxylin – eosin, toluidine blue)		+ toluidine blue
	special staining		
	(Lugol – Calleja, PAS – Calleja)		
Meat sample with flour	general staining	-	+ Calleja
	(hematoxylin – eosin, toluidine blue)		+ 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	+ toluidine blue
		ALERT test	

+ preparation background staining

- examination not performed

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	+	+

+ 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 ⁻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	+	+	+

+ positive

- negative

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	ZS	69	3	72	3
	PS	72	0	72	3

30 g kg ⁻¹	KS	71	0	71	4
	HO	72	0	72	3
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	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
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