Protocol 08	
Monitored ingredient:	wheat protein
Foodstuff:	meat products
Methods:	immunohistochemistry
Version of protocol:	short version

1 Detection Limit

Three samples are sufficient to determine wheat protein at concentration of 0.1 % (and higher) with confidence level greater than 95 %.

2 Time Consumption

Sample preparation: 72 hours
Sample treatment: 9 hours

3 Immunohistochemical Methods

It is necessary to dry the slides on a heating plate for 1 hour. The real examination starts by dewaxing the sections for successful binding between antigen – antibody. The sections are dewaxed in xylene and watered in a descending sequence of alcohols.

3.1 ABC Method

Tab. 1 Immunohistochemical procedure by ABC method

Step No.	Phase	Step	Duration
1	Fixation	Coating slides by a solution: 0,5 g gelatin dissolved in 100 ml warm water + 0,05 g potassium alum + 1 ml poly-L-lysine	
2	Dewaxing	Xylene	7 min.
3		Xylene	7 min.
4		Alcohol 100%	7 min.
5		Alcohol 100%	7 min.
6	Preparation for IHC methods	Bath in tap water	7 min.
7		Bath in distilled water	7 min.
8		Bath in PBS, dilution 1:10	7 min.
9		Bath in citrate buffer, heating in microwave (650W)	5 min.
10		Cooling	20 min.
11		PBS	5 min.

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Step No.	**Phase**	**Step**	**Duration**
12		3 % H2O2 in PBS	20 min.
13		Bath in PBS	5 min.
14		Bath in PBS	5 min.
15		5 ml TBS + 5µl Tween + 0,25 g powdered milk	20 min.
16	IHC methods	Primary antibody – 45 µl per section	1 hour/24°C
17		Bath in PBS + Tween	5 min.
18		Bath in PBS + Tween	5 min.
19		Secondary biotinylated antibody – 45 µl per section	30 min.
20		Bath in PBS	5 min.
21		Bath in PBS	5 min.
22		ABC reagent – 5 ml TBS, 2 drops of reagent A and 2 drops of reagent B	30 min.
23		Bath in PBS	5 min.
24		Bath in PBS	5 min.
25		DAB – 2 drops per section	3 – 5 min.
26		Bath in distilled water	5 min.
27	Staining	Bath in staining solution/s	Depending on the solutions used
28		Rinsing in distilled water	–
29	Dewater	Bath in alcohol	10 min.
30		Bath in acetone	3 min.
31		Bath in xylene I	5 min.
32		Bath in xylene II	5 min.
Staining results		Wheat protein	Brown
		Other ingredients	Depending on the staining used

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Preparation of Solutions
PBS (phosphate buffered saline)
2000 ml distilled water
160 g sodium chloride
4 g potassium chloride
4 g monopotassium phosphate
46.8 g monosodium phosphate
2 pieces of sodium hydroxide

- it is necessary to filter the solution and to adjust its pH to 7.4
- dilute the solution 10x before use

Citrate buffer
2000 ml distilled water
42 g citric acid
18 g sodium hydroxide

- it is necessary to filter the solution and to adjust its pH to 6.0
- dilute the solution 10x before use, check pH on regular basis

Tris buffered saline (TBS)
1000 ml distilled water
1x sack of TBS

- store the solution in the fridge

4 Background Staining

Background staining is used to visualize other structures in the section, to improve contrast between wheat protein and other structures and to improve transparency of the entire section.

The best background staining with good imaging properties is basic staining with toluidine blue which provides good contrast also for qualitative methods (by stereology or image analysis).

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Tab. 2 Background staining by toluidine blue

Step number	Phases	Steps	Times
1	Staining	Toluidine blue	3 – 5 min
2		Rinsing in distilled water	–
3	Dewatering	Bath in alcohol 96%	10 min.
4		Bath in alcohol 100%	3 min.
5	Clearing up	Bath in xylene I	5 min.
6		Bath in xylene II	5 min.

Preparation of Toluidine Blue Solution
100 ml distilled water
0.7 g toluidine blue stain
to 70 ml of this solution add 30 ml glycerol and 1.01 g phenol
- it is necessary to filter the solution and adjust its pH to 7.0
- it is necessary to check the pH before each use

Results
meat – blue with purple-red nuclei
elastic fibers – turquoise
collagen tissue – light salmon pink with blue-violet fibroblasts
wheat protein – brown (light blue-green)
herbal cell walls – fuchsia
starch and neural lipids – no staining

5 Microscopic Methods and Evaluation of Results

Samples are investigated by light microscope with 100x and 400x magnification which is also suitable for photo documentation. It is possible to use stronger magnification for studying certain details. Usually, only qualitative methods is performed. However, quantitative methods is also possible by stereology or image analysis.

Using this immunohistochemical protocol, wheat protein is detected based on its visualization by DAB chromogen (brown) in contrast to stained background (blue). However, this method has its limits in detection of wheat flour, where this staining makes just weak visualization of wheat protein.

For identification of other components, it is recommended to use information presented in literature, samples prepared in a laboratory, schematic pictures and photo documentation.

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6 Documentation

It is recommended to make a laboratory protocol for each sample with the following information:

- number of sample
- date of sampling
- type of product
- producer
- ingredients (if known)
- sample treatment and preparation (fixation, staining, etc.)
- examination results

For clear identification, it is also recommended to label sample container by sample name and number (the same as written in the protocol). Blocks and slides should be labeled in the same way. It is possible to label samples by bar-codes.

All samples with photo documentation are archived.

7 Results

Brown stained wheat protein identified on the basis of their typical spongy structure with small holes and starch residues.

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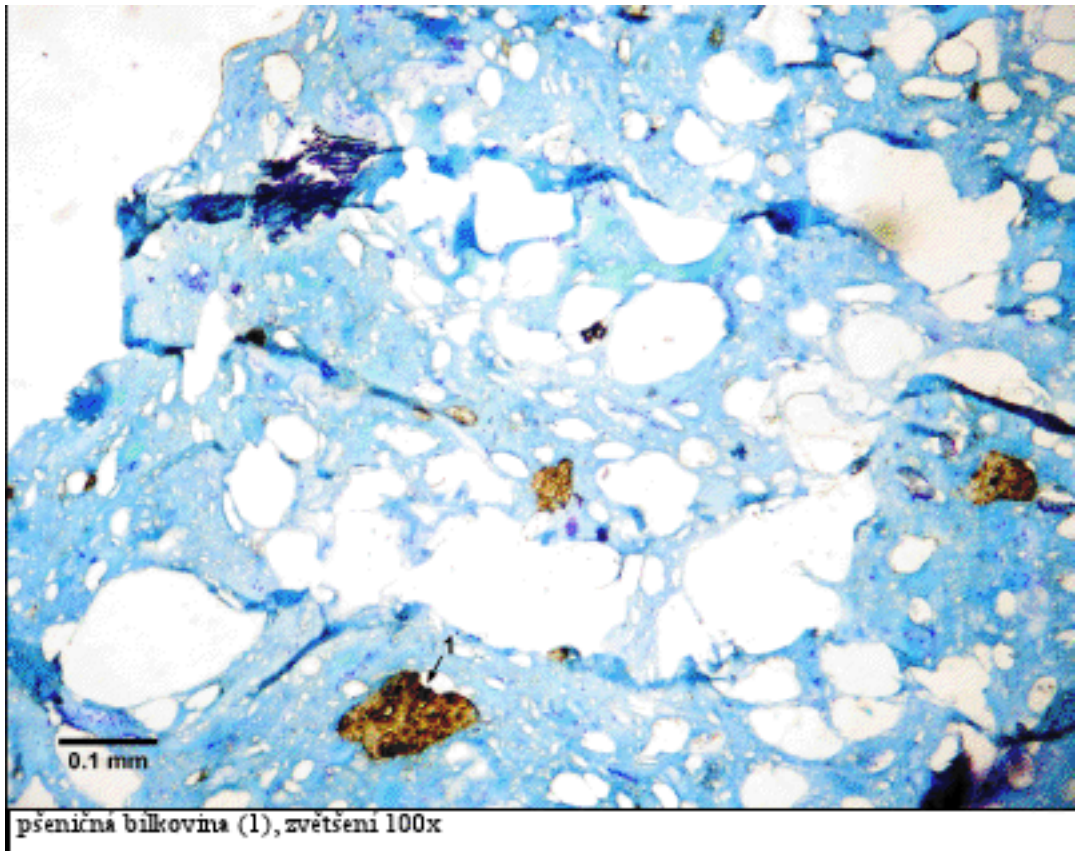
8 Photo Documentation

Description: wheat protein (1), 100x magnified



pšeničná bílkovina (1), zvětšení 100x

9 List of Abbreviations

- ABC ..... avidin biotin complex
- DAB chromogen ..... 3,3´-diaminobenzidine
- TBS ..... trisphosphate buffered saline