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

1 Sample Description

Wheat protein can be detected in samples of different types of meat products using protocol No. 08.

2 Detection Limit

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

3 Time Consumption

Sample preparation: 72 hours
Sample treatment: 9 hours

4 Sampling

4.1 Sample Amount

For the detection it is necessary to take samples in a sufficient amount. Usually, a whole meat product (one package) or at least 30 g is taken.

4.2 Sampling

Meat products are taken from retail market. It is also possible to investigate chilled or frozen samples delivered directly from the manufacturer (including meat work).

4.3 Sample Fixation

Samples are fixed in 10% water solution of neutral formaldehyde for 24 hours as soon as possible after sampling. Frozen samples are processed after slow thawing (by 27°C).

Principles of Fixation

- samples must be inserted in a special box or in a gauze in a fixative solution as soon as possible;
- sample size should not exceed 1 cm;
- the amount of fixative solution must be at least 20 – 50 times greater than the volume of the fixed sample;

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- the sample must be accessible to the fixative solution from all sides, if necessary, it is possible to put the sample on cotton wool or filter paper.

5 Material and Equipment

5.1 Chemicals and Solutions

- alcohol
- potassium dichromate
- formol
- citric acid
- sodium chloride
- potassium chloride
- monopotassium phosphate
- monosodium phosphate
- sodium hydroxide
- hard paraffin grated
- potassium alum
- solution-based acrylic resin
- tap water
- distilled water
- xylene pure
- xylene p. a.
- hydrogen peroxide 30%
- toluidine blue stain
- 3,3´-diaminobenzidine
- antibody diluent
- trisphosphate buffered saline
- TWEEN R 20
- polyclonal anti-wheat antibody isolated from a rabbit
- avidin biotin complex with secondary anti-rabbit antibody conjugated with biotin

5.2. Equipment

(uses strictly in accordance of the appropriate manual)
- autotechnicon
- microtom
- fume hood
- staining cuvettes
- micropipette 10–100 µl
- micropipette 100–1000 µl
- micropipette 0,1–2 µl

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- micropipette 1–10 ml
- mini centrifuge MPW 15
- distillation apparatus
- scales
- pH meter
- fridge
- freezing box
- hotplates
- thermostat
- moistened cell

5.3 Laboratory Tools

- gauze
- tweezers
- scalpel
- knife
- protective gloves
- plastic Pasteur pipette 1 ml
- plastic Pasteur pipette 3,5 ml
- cutting mat
- embeading cell
- markers
- liquid blocker super pappen
- thermometers
- cotton wool
- cotton swabs
- filtration paper
- magnetic stirrer

5.4 Laboratory Glass

- pipette 5ml
- pipette 10ml
- beaker 200 ml
- beaker 2000 ml
- slides for immunohistochemistry
- cover slips
- funnels

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6 Sample Treatment and Preparation

6.1 Preparation of Paraffin Blocks

Samples (5 g) are fixed in 10% water solution of neutral formalin for 24 hours. After fixation, the samples are dewatered in ascending sequence of alcohol and embedded into paraffin blocks (table 1 and 2).

Tab. 1 Manual preparation of paraffin blocks

Step No.	Chemicals	Duration
1	Water	30 min
2	Alcohol 20%	30 min.
3	Alcohol 40%	60 min.
4	Alcohol 50%	60 min.
5	Alcohol 70%	120 min.
6	Alcohol 80%	Per night
7	Alcohol 96%	60 min.
8	Alcohol 100%	120 min.
9	Methyl salicylate I	60 min.
10	Methyl salicylate II	60 min.
11	Methyl salicylate III	Per night
12	Xylene I	15 min.
13	Xylene II	15 min.
14	Xylene III	15 min.
15	Paraffin I	120 min.
16	Paraffin II (p. a.)	120 min.
17	Paraffin III (p. a.)	Per night
18	Embedded into paraffin blocks	4th day

Tab. 2 Preparation of paraffin blocks in autotechnicon

Step No.	Chemicals	Duration
1	Water	30 min
2	Alcohol 50%	40 min.

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3	Alcohol 70%	20 min.
4	Alcohol 96%	60 min.
5	Alcohol 96%	100 min.
6	Alcohol 96%	60 min.
7	Alcohol 100%	60 min.
8	Alcohol 100%	60 min.
9	Acetone	20 min.
10	Xylene I	20 min.
11	Xylene II	20 min.
12	Paraffin I	180 min.
13	Paraffin II (p. a.)	12 hours
14	Embed into paraffin blocks	

6.2 Embedding into Paraffin Blocks

We recommend preparing at least one block for each sample. For the purposes of embedding samples into paraffin blocks, commercial medium based on paraffin in combination with bee wax is used. This medium is insoluble in water. Samples saturated with paraffin are embedded in embedding cells by an embedding line. After cooling down, these blocks are prepared for cutting.

6.3 Cutting the Blocks

Tissues embedded in paraffin blocks are cut to 4 μm sections by a microtome. The samples are cut for immunohistochemical examination according to the procedure described in the following scheme. The sections are then spread on the water surface and mounted to slides.

Cutting Scheme
section 1 – cut off 50 μm – section 2 – cut off 50 μm – section 3 – cut off 50 μm – section 4 – cut off 50 μm – section 5 – cut off 50 μm – section 6

6.4 Immunohistochemical Method

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.

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6.5 ABC Method

Tab. 3 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.
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 procedure	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.

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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

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

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

- store the solution in the fridge

6.5.1 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 method (by stereology or image analysis).

Tab. 4 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)

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herbal cell walls – fuchsia
starch and neural lipids – no staining

6.6 Mounting of Stained Slides

Stained sections are mounted between a slide and a cover glass with a suitable mounting medium which does not interfere with the color. Usually, synthetic resins insoluble in water are used, so it is necessary to dewater the sections in an ascending sequence of alcohol and xylene.

Process for Manual Mounting

1. put a drop of mounting medium on the margin of the slide
2. place a cover glass on the margin of the slide under the angle of 45°
3. carefully and slowly move the cover glass in order to avoid formation of bubbles
4. put slides in thermostat with 60°C for the night
5. carefully clean the edges using alcohol and a razor

It is also possible to use a mounting automate.

6.7 Microscopic Method 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 method is performed. However, quantitative method 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.

6.8 Documentation

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

- number of sample
- date of sampling

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- 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.

6.9 Results

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

7 Photo Documentation

Description: wheat protein (1), 100x magnified

8 List of Abbreviations

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