Protocol 09	
Monitored ingredient:	wheat protein
Foodstuff:	meat products
Methods:	immunohistochemistry
Version of protocol:	short

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	Sample treatment
24 hod.	24 hod.

3. Immunohistochemical procedure

It is necessary to dry the slides on a heating plate for 1 hour. Use superfrost + slides. The procedure for paraffin sections 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. The procedure for frozen sections starts after fixation with acetone.

6.5 ABC Method

4. Tab. 3 Immunohistochemical procedure by ABC method

Step No.	Phase	Step	Duration
1	Dewaxing	Xylene	7 min.
2		Xylene	7 min.
3		Alcohol 100%	7 min.
4		Alcohol 100%	7 min.
5	Preparation for IHC examination	Bath in tap water	7 min.
6		Bath in distilled water	7 min.
7		Bath in PBS, dilution 1:10	7 min.
8		Antigen retrieval in citrate buffer, heating in microwave (650W)	5 min.
9		Cooling	20 min.
10		PBS	5 min.
11		3 % H_2O_2 in PBS	20 min.
12		Bath in PBS	5 min.
13		Bath in PBS	5 min.
14		Block of unspecific bind by 5 ml TBS + 5µl Tween + 0,25 g powdered milk	20 min.

15	IHC examination	Primary antibody – 45 µl per section. Anti-soy antibody isolated from a rabbit 8,0mg/ml, SigmaAldrich	8 hour/8°C
16		Bath in PBS	5 min.
17		Bath in PBS	5 min.
18		Secondary anti-rabbit biotinylated antibody – 45 µl per section	30 min.
19		Bath in PBS	5 min.
20		Bath in PBS	5 min.
21		ABC reagent – 5 ml TBS, 2 drops of reagent A and 2 drops of reagent B	30 min.
22		Bath in PBS	5 min.
23		Bath in PBS	5 min.
24		DAB – 2 drops per section	1 – 3 min.
25		Bath in distilled water	5 min.
26	Staining	Bath in staining solution/s	Depending on the solutions used
27		Rinsing in distilled water	–
28	Dewater	Bath in alcohol	10 min.
29		Bath in acetone	3 min.
30		Bath in xylene I	5 min.
31		Bath in xylene II	5 min.
Staining results		Soya protein	Brown
		Muscle tissue	green
		Elastic tissue	yellow
		Collagen tissue	blue
		Starch and fat	don´t stained

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 stock 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 stock 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
- if is necessary filter the solution (in the presence of precipitate)

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 modified Calleja staining which provides good contrast also for qualitative examination (for stereology or image analysis).

Tab. 4 Background staining by modified Calleja

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

Preparation of modified Calleja Solution

100 ml distilled water
1 g indigocarmine
200 ml picric acid

Results

Soya protein – brown
Muscle tissue – green
Elastic tissue – yellow
Collagen tissue – blue
Starch and fat – don´t stained

6.6 Mounting of Stained Slides

Stained sections are mounted between a slide and a cover slide 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 for solakryl BMX

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 Examination 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 examination is performed. However, quantitative examination is also possible by stereology or image analysis.

Using this immunohistochemical protocol, soya protein is detected based on its visualization by DAB chromogen (brown) in contrast to stained background (green etc.).
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
- type of product
- producer
- ingredients (if known)
- sample treatment and preparation (fixation, staining, etc.)
- examination results

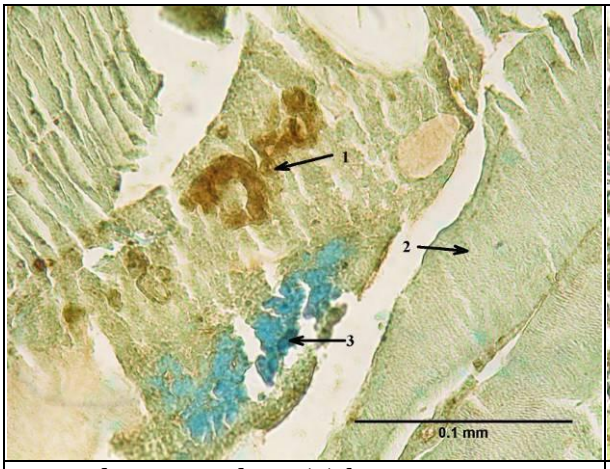
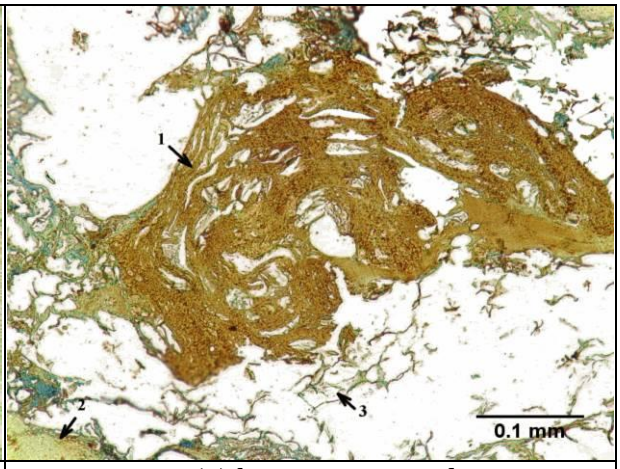
For clear identification, it is also recommended to label sample container by sample name and number (the same as written in the protocol). Blocks and slides should be labeled in the same way. It is possible to label samples by bar-codes.

All samples with photo documentation are archived.

6.9 Results

Brown stained soya protein identified on the basis of their typical spongy-shaped, sickle-shaped, moon-shaped, or circular-shaped corresponding to individual protein types. The structure can be with or without small holes. Texturized soya proteins can be identified on the basis of their typical fibrous structures with common structural elements, such as palisade and goblet cells.

5. 7 Photo Documentation

	
Heated meat product, (1) brown soya protein, (2) muscle, (3) collagen tissue, magnification 100 x.	Dry sausage, (1) brown texturized soya protein, (2) muscle (3) residues of fatty, magnification 100x.

6. 8 List of Abbreviations

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