Protocol 09	
Monitored ingredient:	soya protein
Foodstuff:	meat products
Methods:	immunohistochemistry
Version of protocol:	full

1 Sample Description

Soya protein can be detected in samples of different types of meat products using protocol No. 09. The protocol is usable for frankfurters, sausages, salami and dry sausages

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	Sample treatment
24 hod.	**24 hod.**

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

Vzorky se co nejdříve po odebrání fixují v 10 % roztoku neutrálního formolu, nejlépe přímo na místě odběru. Zmrazené vzorky se zpracovávají po rozmrazení.

Samples are fixed in 10% water solution of neutral formaldehyde for 24 hours as soon as possible after sampling. Frozen samples are air dried. Immediately before staining, are frozen samples fixed with could acetone (-18°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;
- 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, 96%
- - potassium dichromate
- - formaldehyd 36-38% p.a.
- - citric acid
- - sodium chloride
- - potassium chloride
- - monopotassium phosphate
- - monosodium phosphate
- - sodium hydroxide, p.a.
- - hard paraffin grated, in Vitro melting 56°C
- - potassium alum
- - solution-based acrylic resin
- - tap water
- - distilled water
- - solakryl BMX
- - xylene pure
- - xylene p. a.
- - hydrogen peroxide 30%
- - indigocarmine
- - picric acid, 50-60%. p.a.
- - 3,3´-diaminobenzidine, Dako Cytomation (Dako Liquid DAB+substrate)
- - antibody diluent, Dako Cytomation
- - trisphosphate buffered saline, SigmaAldrich
- - TWEEN R 20, BioXtra
- - polyclonal anti-soy antibody isolated from a rabbit 8,0mg/ml, SigmaAldrich
- - avidin biotin complex with secondary anti-rabbit antibody conjugated with biotin (PK-6100), Vector laboratories

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
- - micropipette 1–10 ml
- - mini centrifuge MPW 15

- - distillation apparatus
- - Analytical weighing scale, accuracy 0,01 g, range 0-750g
- - pH meter
- - fridge
- - freezing box
- - hotplates accuracy 0,5°C , range 0-100°C
- - thermostat 54°C
- - moistened cell

5.3 Laboratory Tools

- - tweezers
- - scalpel
- - knife
- - cutting mat
- - protective gloves
- - tissue Cassettes
- - plastic Pasteur pipette 1 ml
- - plastic Pasteur pipette 3,5 ml
- - plastic eppendorf tubes 1,5 ml
- - embeading cell
- - markers, liquid blocker super PapPen
- - thermometers
- - filtration paper
- - magnetic stirrer

5.4 Laboratory Glass

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

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.
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

For each sample are prepared four blocks. 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 – cut off 50 μm – section 7 – cut off 50 μm – section 8 – cut off 50 μm – section 9

6.4 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

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

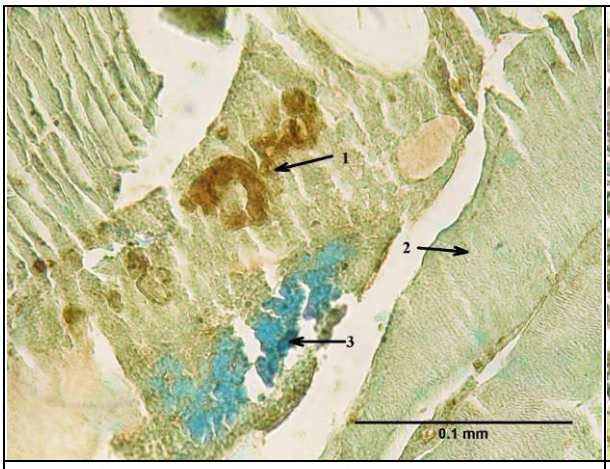
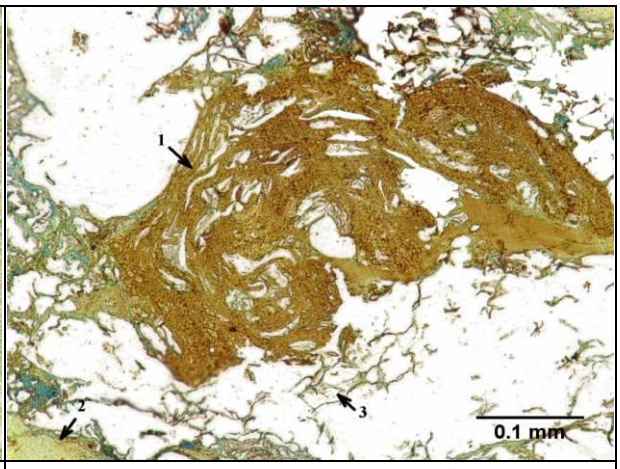
For clear identification, it is also recommended to label sample container by sample name and number (the same as written in the protocol). Blocks and slides should be labeled in the same way. It is possible to label samples by bar-codes.

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.

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.

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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