Protocol 01	
Monitored ingredient	Bone fragments
Foodstuff	Meat products
Examination	Histochemistry, Alizarin red stainig
Short protocol/full version	Full version

1 Sample Description

According to this method are examined samples of different types of meat products and samples of mechanically separated meat (MSM).
By using histochemical staining we can prove the presence of bone fragments and on this basis we can pronounce suspicion of using of mechanically separated meat as a raw material in meat product.

2 Detection Limit

The limit of detection is not necessary define in the volume percentage, because it is ingredient which is rare in meat products. A common finding is only in case of mechanically separated meat, which includes bone fragments. Certificate of using mechanically separated meat is considered to finding 3 bone fragments in 10 histological sections.

3 Time Consumption

Sample preparation	Sample treatment
24 hours	41 minuts

4 Sampling

4.1 Sample Amount

For the detection it is necessary to take samples in a sufficient amount.
PIcked up:

mechanically separated meat	at least 50 g
meat product	whole meat product (one package)

4.2 Sampling

Sampling of MSM is done in company, where mechanically separated meat is produced on various types of production equipment . We can also process chilled or frozen samples provided by the producer.
Meat products are taken from retail market.

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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 thawing.

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^3,
- 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
- methylsalicylate
- alizarin red S
- formol
- indigocarmine
- acetic acid
- acid picric
- hard paraffin grated
- buffers: pH 4 phtalate, pH 7 Phosphate
- sodium hydroxide
- xylene pure
- xylene p. a.

5.2 Equipment

(uses strictly in accordance of the appropriate manual)

- autotechnikon
- microtom
- fume hood
- staining cuvettes/staining automat
- distillation apparatus
- scales

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- pH meter
- thermostat
- hotplates

5.3 Laboratory Tools

- gauze
- tweezers
- scalpel
- knife
- cutting mat
- embeading cell
- protective gloves
- markers
- thermometers
- cotton wool
- cotton swabs
- filtration paper
- magnetic stirrer

5.4 Laboratory Glass

- pipettes
- beaker 200 ml
- slides for histochemistry
- 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).

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Table 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	4^{th} day

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Table 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

We recommend preparing at least four blocks 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 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

block A

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**

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block B

section 7 – cut off 50 μm – **section 8** – cut off 50 μm – **section 9** – cut off 50 μm – **section 10** – cut off 50 μm – **section 11** – cut off 50 μm – **section 12**

block C

section 13 – cut off 50 μm – **section 14** – cut off 50 μm – **section 15** – cut off 50 μm – **section 16** – cut off 50 μm – **section 17** – cut off 50 μm – **section 18**

block D

section 19 – cut off 50 μm – **section 20** – cut off 50 μm – **section 21** – cut off 50 μm – **section 22** – cut off 50 μm – **section 23** – cut off 50 μm – **section 24**

6.4 Stainig

Sectiones are necessary before staining get rid of paraffin. Paraffin sections dissolves in the solvent (xylene) and through ethanol are the incisions converted into water because histological dyes are mostly soluble in water. We can dyed by hand in special cuvettes or in the staining automat. Dyes and dye mixtures are applied according to the procedure described below.

Alizarin red

Objective: highligting bone fragments

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Procedure:

Getting rid of paffin	xylen.....	10 min
	100 % alcohol + ether (2/3+1/3).....	10 min
1% acetic acid.....		Wash
Distilled water.....		Wash
Solution acid picric and alizarin.....		5 minut
Distilled water.....		Wash
Indigocarmine.....		3 min
Distilled water.....		Wash
Dewatering (alcohol)	96%.....	Wash
	100%.....	Wash
Xylene I (pure)	.....	5 min
Xylene II (p. a.)	.....	5 min

Preparation of Solutions:

Acid picric and alizarin:

0,5 g	alizarin red S
50 ml	distilled water
50 ml	1,2% acid picric

- Add a picric acid in dissolved alizarin in distilled water and stir. Using sodium hydroxide to adjust pH from 4.3 to 4.5. Filter and add thymol (for preservation).

Indigocarmine:

0,2 g	indigocarmine
80 ml	distilled water

Stainig results:

bone fragments	- yellow/red
fibrous connective tissue	- blue

6.5 Mounting of Stained Slides

Stained sections are mounted between a slide and a cover slip with a suitable mounting medium which does not interfere with the color. Usually, used synthetic resins are insoluble

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in water, 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 slip 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.

7 Microscopic Examination and Evaluation of Results

The stained sections are examined by the light microscope with a lower magnification (e.g. 32x or 40x), for the study of detail is used higher magnification. Usually, only qualitative examination is performed. Describes the presence of different types of tissues in the examined samples. It is possible to focus only on identification of selected tissues highlighted by special staining. The identification of tissues of animal and vegetable origin must be based on data from the literature. For comparison use samples prepared in the laboratory and also the schematic pictures and photos from the literature.

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.

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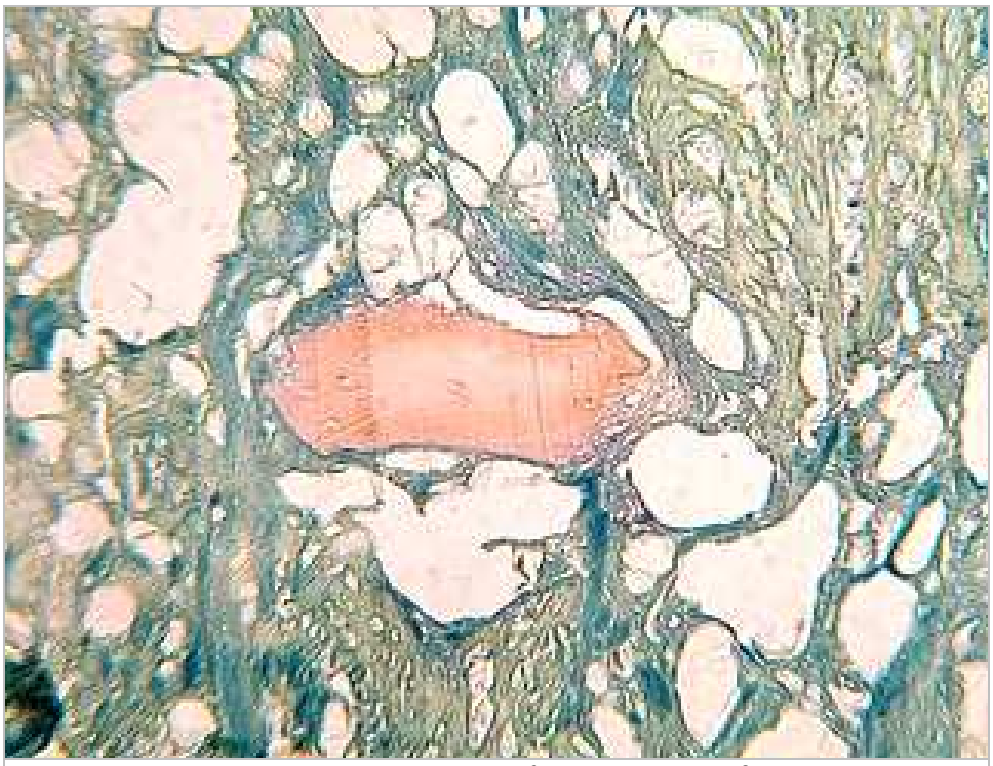
9 Results

Bone fragments are stained from yellow to red.

10 Photo Documentation



Vienna sausage, stainig AC, bone fragment, magnification 250x

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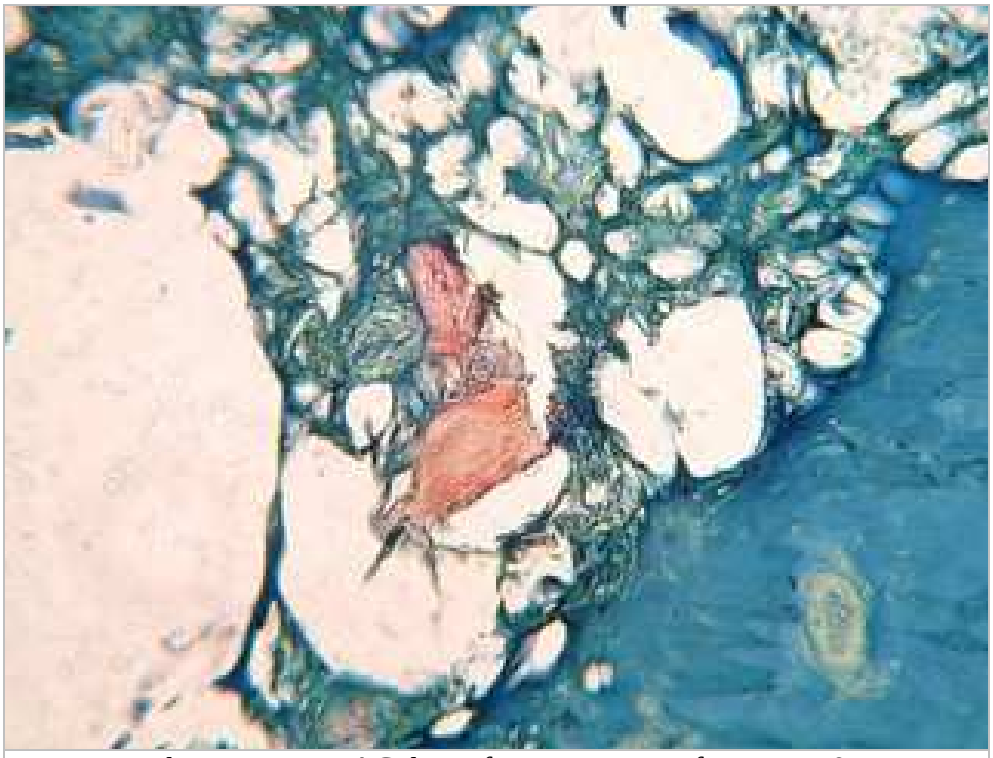

Kabanos, stainig AC, bone fragment, magnification 250x

11 List of Abbreviations

AC – alizarin red