The benefits of applying the microscopic examination in the analysis of meat products

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Abstract
The microscopic examination of meat products allows the identification of tissue structures and, to a certain extent, of the unauthorized content of plant and animal origin, the detection of parasites, the quality evaluation of the meat having undergone processes of freezing and thawing, the detection of muscle degeneration, the detection of foodstuffs adulteration and of the dangerous ingredients in meat products. The purpose of this study was to emphasise the importance of the results attained by microscopic evaluation of the integrity and quality of the meat used for meat products, the detection of the constituents of animal and vegetal origin, of the non-authorized tissues in the analysed products using routine staining as well as special staining. A total of 22 samples from different categories of meat products, represented by: boiled and smoked products (n = 5), cooked and double-smoked products (n = 2), raw-dried meat products (n = 6), baked and smoked meat products (n = 3), smoked meat products (n = 6), were randomly purchased from the commercial network within their validity period. All samples were processed using the routine paraffin inclusion technique, initially stained with HE (haematoxylin - eosin), special Masson's trichrome stain (connective tissue) and GMS II (Grocott Methenamine Silver, for the revealing of fungi). Microscopically, in the examined sections, there were highlighted different types of connective tissue (adipose and fibrous) in all of the samples, serous glandular tissue in baked and smoked meat products and in raw-dried meat products, visceral fragments (kidneys) in baked meat products and in raw-dried meat products, parasitic structures (Sarcocystis spp.) in baked and smoked meat products and in smoked meat products, fungal fragments in baked and smoked meat products and in smoked meat products, their presence being unauthorized. We consider that such an analysis, if appropriately performed and interpreted, can provide objective information in order to verify the compliance of these products with the legislation in force and to ensure the accurate composition and the integrity of these foods. By means of routine microscopic examination, the HE technique, non-compliant structures of animal origin were identified: visceral fragments and parasitic structures, Masson's trichrome stain method allows a better highlighting of the connective tissue, and the GMS 2 technique can be used successfully for highlighting the fungal structures in meat products..
Keywords: *microscopic techniques, quality, meat products.*

Introduction

Microscopic methods, along with chemistry, immunochemistry and molecular biology, still represent an alternative and, in some cases, a less costly alternative to food products examination and control. The history of food microscopy dates back to 1850, when Hassel used the microscope to distinguish chicory from coffee (Pospiech M, et al, 2011). In meat products, microscopic techniques have been used since 1910 in order to detect their contamination or intentional adulteration (Tremlova B, 2003). In Europe, for instance, Clinquart presented an overview of research in the same field, food analysis. 75% of the studies were performed on meat products, 25% of which were made using histology as a discipline (Clinquart et al, 2006). This topic has been addressed by many researchers such as: Aguilera: Microstructural Principles of Food Production and Analysis, Flint: Microscopy of Food, (Flint O,1994) Tremlová: Histology of Food. (Tremlová B, 2006)

Currently, various bioimaging techniques are available for the microscopic determination of food components. Commonly, the most used method is optical microscopy. This allows the identification of all structures through their morphological characteristics. In addition, special staining allows the 'selected' structures to be highlighted, with colors different from those of the other parts of the product under examination. (Pospiech M, et al., 2011).

The interest in identifying the composition has increased and many people are concerned about the meat and meat products they consume (Ballin NZ, 2010). In this respect, several studies having the microscopic examination as a method of study were published. Among these , Prayson studied the composition of hotdogs (Prayson BE, 2008a), hamburgers (Prayson B. 2008b) and other meat products (Malakauskiene S, 2016, Ghisleni G, et al., 2010). Using the same technique (Abdel Hafeez H et al, 2016) there are detected lung, ruminant stomach, large elastic blood vessels, myocardium, cartilage, spongy bone and lymphatic tissue (spleen) in meat sandwiches. Avinee et al. examined six samples of Merguez and Chipolatas sausages.

All samples contained fragments of fibrotenous tissue and parts of bone and cartilage. It is noteworthy that no nervous tissue was found in the evaluated samples (Avinee G, et al., 2010). In the same context, the microscopic examination allowed the identification of parasites in fresh fish samples, in food intended for processing (fish paste) and in finished products (smoked salmon). The method allowed the identification of two important parasites *(Anisakis simplex and Kudoa spp*). (Delphine MO.et al., 2010).

For this reason, the microscopic examination was adopted in some developed countries as a complementary method of evaluating product integrity.(Pospiech M, et al., 2011).There are many means to process and prepare samples for microscopic examinations today, along with a variety of investigative techniques. For example, in the Czech Republic the microscopic analysis of foodstuff is monitored by a team of experts from the Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno. These researchers combine microscopy methods with state-of-the-art methods from other disciplines such as immunohistochemistry, image analysis and stereology ([Pospiech M, Lukaskova ZR, Tremlova B, et al., *Microscopic methods in food analysis.* MASO Int BRNO. 2011)

Materials and methods

22 samples from the commercial network were included in the study during the period of validity and were represented by: boiled and smoked products (n = 5), boiled and double-smoked products (n = 2), raw-dried meat products, (n = 6), baked and smoked meat products (n = 3), smoked meat products (n = 6). From the microscopic examination viewpoint, the samples were subjected to the routine method by inclusion to paraffin using a histoprocessor, sectioned and stained by conventional HE (hematoxylin - eosin) technique, and special Masson's trichrome stain (connective tissue) and GMS II (Grocott Methenamine Silver for the detection of fungi). The sections were examined with the Olympus BX 41 Microscope with integrated computer photographing system.

The characteristics of the sample, including: sample type and the recipe are recorded in table1.

Table 1. Label composition of analyzed samples.

No	Sample type	Product category by processing method	Label Composition
1.	Sausage with spices	Boiled and double-smokedmeat products (n=2)	pork, salt, spice (pepper, garlic, basil) food additive, preservative, sodium nitrate, edible natural membrane
2.	Highly seasoned sausage		pork, fat, water, spices, salt, stabilizator, antioxidant, ascorbic acid, sugar, monosodium glutamate, soy vegetable protein, animal protein, preservative: sodium nitrite, carmine, edible membrane
3.	Semi-smoked pork sausage	Boiled and smoked meat products products (n=5)	minced pork meat, water, lard, vegetable protein from soy, salt, spices, antioxidant (ascorbic acid, ascorbate sodium), monosodium glutamate), acidity regulator (citric acid), extracts form spices, flavorings, preservatives (sodium nitrite),
4.	Semi-smoked pork sausage		minced pork meat, water, fat, soy protein, spices, sugar, dextrose, flavor stabilizers sodium diphosphate, sodium polyphosphates, antioxidant, sodium ascorbate, preservative: sodium nitrite,
5.	Beer-sausages		beef, pork, rind, lard, soy vegetable protein, salt,spices, preservative: sodium nitrite,dextrose.
6.	Chicken pastrami		chicken breast 91% without bone, soybean protein, potato starch, water, salt, spices, flavor, sugar, sodium phosphate stabilizers, thickener, carrageenan, anti-agglomerant (calcium phosphate), antioxidants, sodium acidity, flavor enhancers
7.	Turkey pastrami		turkey leg, marine salt, natural spices, sugar, preservative (sodium nitrite)
8.	Sibiu Salami	Raw dried products (n=6)	pork, fat, salt, sugars, spices, ascorbic acid, sodium nitrite
9.	Sibiu Salami		pork, fat, salt, sugars, spices, ascorbic acid, brandy 0.4%, sodium nitrite
10.	Ardelenesc Salami		unknown recipe
11.	Sibiu Salami		pork, fat, salt, sugars, spices, ascorbic acid, sodium nitrite, starter culture
12.	Sibiu Salami		pork, fat, salt, dextrose, spices, sodium ascorbate, sodium nitrite
13.	Sibiu Salami		pork, fat, salt, dextrose, spices, ascorbic acid, sodium ascorbate, sodium nitrite, starter culture

14.	Traditional pork sausage	Baked and smoked (n=3)	pork, sodium nitrate, water, garlic, spice extracts, sugars (dextrose), flavour enhacer (monosodium glutamate and antioxidants)
15.	Traditional pork sausage		pork, sodium nitrate, water, garlic, spice extracts,
16.	Lamb sausage		lamb sausage unknown recipe
17.	Lamb pastrami	Smoked meat products (n=6)	unknown, traditional system from small producers
18.	Beef pastrami		young beef leg, coarse salt, bay leaves, thyme, white onion, hot chili
19.	Pork Pastrami		fresh lean pork leg, salt, bay leaves, thyme, hot pepper, onion, garlic
20	Beef pastrami		beef leg, salt, natural spices, sugar, preservative (sodium nitrite)
21	Pork Pastrami		pork leg 96%, salt, pepper, thyme, hot pepper, garlic, preservative (sodium nitrite)
22	Smoked chop		Boneless loin ingredients: salt, heat treated by smoking.

Results and discussions

The tissue types identified in each sample are summarized in Table 2. The most observed tissue of connective tissue (adipose and fibrous) (n=22), was detected in all samples. Among 22 studied samples, the observed unauthorized tissues were included: organs (kidneys) (n=1), Serous glandular tissue (n=3), *Sarcocystis spp*.(n=5), lymphoid tissue (n=2), fungal structure (n=3)

Table2. Tissues detected in meat products based on microscopical examination

Sample Type	Sarcocistis spp.	Serous glandular tissue	Organs (kidneys)	Adipose Tissue	Fibrous Tissue	Lymphoid Tissue	Fungal Structure
boiled and double-smoked products (n=2)	0	0	0	2	2	0	0
boiled and smoked products (n=5)	1	1	0	5	5	2	1
smoked meat products (n=6)	3	0	1	6	6	0	2
raw-dried meat products (n=6)	0	1	0	6	6	0	0
baked and smoked meat products (n=3)	1	1	1	3	3	0	0

Microscopical Findings

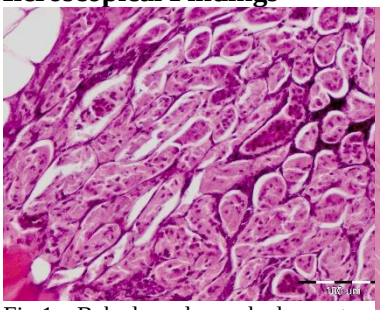

Fig.1. Baked and smoked meat products - Lamb Sausage - (kidney), (ob. 20, HE stain)

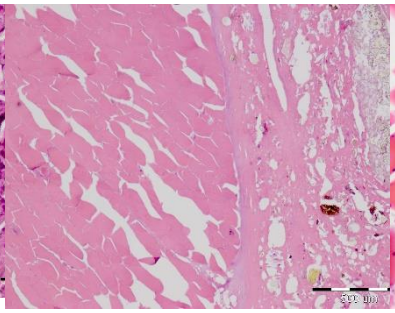

Fig.2. Boiled and smoked meat products - Turkey Pastrami – microscopical examination reveals muscle fibres with adipose tissue, and connective tissue (ob.10, HE stain)

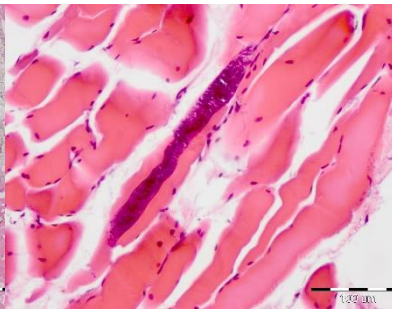

Fig.3. Baked and smoked meat products -
Lamb Sausage, intracellular parasite with a *Sarcocystis spp*. morphology, (ob.10, HE stain)

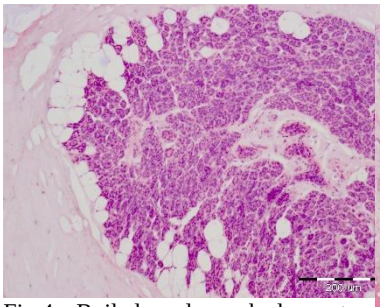

Fig.4. Boiled and smoked meat products- Pork Sausage with Serous glandular tissue, (ob. 10, HE stain)

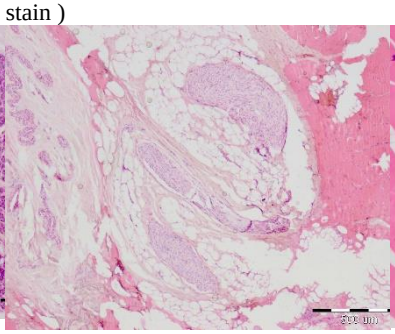

Fig.5. Raw dried products - Salami: microscopical examination reveals muscle fibers with homogenous eosinophilic to amphophilic sarcoplasm, nerve threads, conjunctive tissue and rare adipocytes, (ob.4, HE stain)

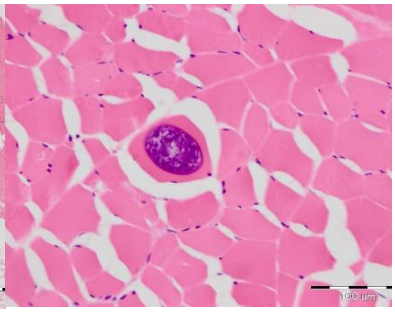

Fig.6. Smoked meat products -Beef Pastrami: in skeletal muscle the *Sarcocystis spp.* appearas basophilic bodies,round, bordered by a radial fairly thick wall (ob.20, HE stain)

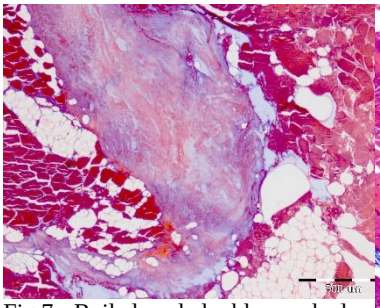

Fig.7. Boiled and double smoked meat products. Pork sausage with spices Connective tissue in abundant amounts with a collagen aspect fibres and with intense blue colour evidentiated by the special Masson's trichrome stain (ob. 10, Masson's trichrome)

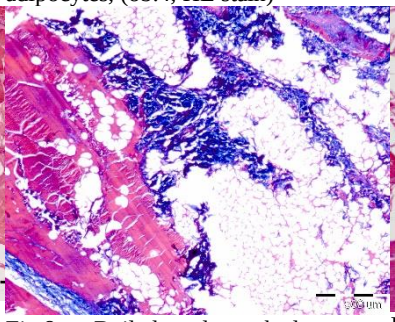

Fig.8. Boiled and smoked meat products.Pork sausage evidentiated by the special Masson's trichrome stain (ob. 10, Masson's trichrome)

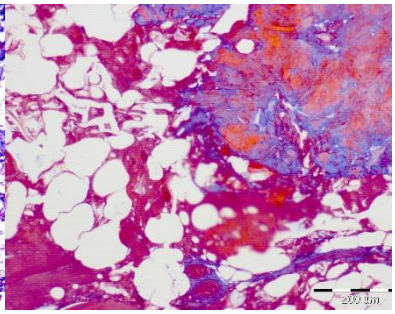

Fig.9. Baked and smoked meat products. Pork sausage connective tissue in a moderate amount, intensively colored blue areas with poorly conserved cellular histological architecture evidentiated by the special Masson's trichrome stain (ob. 10, Masson's trichrome)

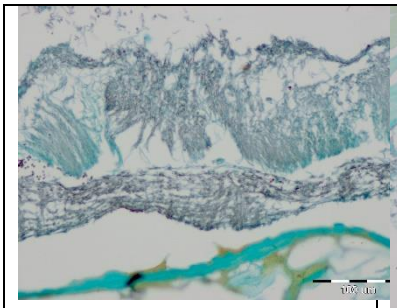

Fig.10. Baked and smoked meat products -Pork Sausage - structuri fungice localizate la periferia și în grosimea fragmentelor tisulare evidențiate prin colorația specială (ob. 20, GMS II)

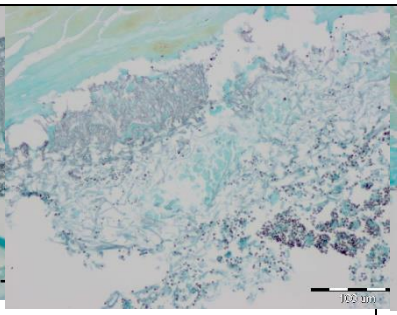

Fig.11. Boiled and smoked meat products - Chicken pastrami- the examined tissue fragments present an intensely oxyphil, filamentous mass with a tendency to penetrate the tissue (evidentiated by special staining (ob. 20, GMS II).

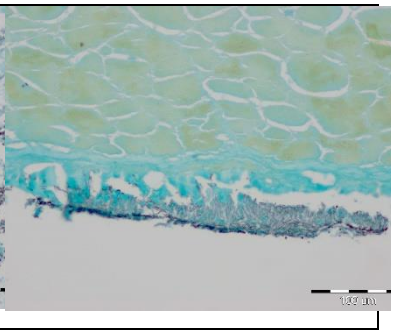

Fig.12. Smoked meat products - Beef pastrami - the periphery of the examined tissue fragments it is occupied by numerous microorganisms with fungal structures with a constant tendency to penetrate the depths of the muscular tissue, confirmed by special staining (ob. 20, GMS II)

The commonly used staining methods are hematoxylin-eosin (red shades) (fig.1, - fig.6,) normally used to highlight most morphological animal and plant components (shape, size and mutual cellular configuration, presence of crystals, granules, or other elements) in the meat products structures.

In some sections of raw dried products (salami), different tissues were evidentiated: striated muscle tissue, various types of connective tissue, adipose tissue in abundance, vascular structures and nerve fibres, in other sections of the same category of product, vegetal structures were revealed with morphology and tinctorial properties different from that of animal tissues. In sections of raw dried products stained by conventional haematoxylin-eosin, the homogeneous aspect of muscle fibers and , in an inconstant manner, their distance from the endomisium were observed, the appearance is associated with the dehydration process after treatment with salt.

The products from the meat processing industry are not exclusively composed of materials of animal origin. A simple microscopic observation with conventional H & E (hematoxylin and eosin) staining makes it easy to identify constituents of plant origin in their traditional form (Pospiech M, et al, 2011). Vanha J, et al., state that the identification of constituents in meat products, combined with an estimate of thier actual quantity, makes it possible to monitor the quality of meat products using the same staining (Vanha J, et al., 2011).

Meat products such as salami, sausages are prone to fraudulent practices. In this study in the microscopic examination of the products of the category: boiled and double-smoked products, boiled and smoked products, baked and smoked meat products, different tissues were found: connective (adipose and fibrous) in (fig.2), in (fig.5), however, a wide range of unauthorized tissue was detected including: glandular serous tissue (fig.4), lymphoid tissue, visceral fragments (kidney) in fig. 1, parasitic structures (*Sarcocystis spp.)* in (fig.3) and (fig.6).

Similarly, Atasever et al (1999) reported finding parts of organs that should not be included in the technological process of fermented sausages, according to the Regulation and Standard, such organs were found in eighteen (37%) of the fourty-eight fermented sausage samples they procured from the market . In the 50 sausage samples examined by Erdoğrul (2002), cartilage and bone tissue were found in 24%, adipose tissue in 50%, connective tissue in 10% and nerve tissue in 16%. (Erdoğrul ÖT, 2002).

In another study, 30 samples from three different types of sausages were assessed by microscopic examination. The most frequently observed additive tissues consisted of chicken skin, hyaline cartilage, peritoneal fat and kidney (Sepehri Erayi, 2008).

Sadeghi et al. used the histological method to examine 720 sausage samples in which unauthorized tissues such as adipose tissue, myocardium , cartilage, esophagus, salivary glands (Sadeghi et al., 2011) were identified.

A similar histological evaluation of hamburgesr, Kabab Loghme and minced meat , marketed in Tehran, Iran showed the presence of some unauthorized tissues such as blood vessels, nerves, cartilage, adipose and plant material (Sepehri Erayi, 2008) facts that are in accordance with our findings.

What attracted attention was the presence of round, intensely basophilic structures, identified in muscle fibers as *Sarcocystis spp*., in samples of the category of smoked meat (pastrami) and of the category of baked and smoked products. *Sarcocystis spp.* is an intracellular parasite in mammals, which represents a considerable infection rate especially in sheep and cattle. Human infection with *Sarcocystis* may be related to the consumption of raw, unprepared meat or meat products containing the enclosed/encysted parasite (Dehkordi ZS, et al., 2017).
The results showed that more than 80% of the tested samples were infected with Sarcocystis. The rate of infection in sausages and hamburger was 83.33% and 87.5%, respectively; the samples were treated with Giemsa staining and observed under the optical microscope (Guelmamene R, et al., 2018)

One disadvantage of basic staining is that the individual components are presented in different shades of a single color (Fig. 2.5.6). Also, if the product structure has been disturbed during processing, then identification can not be done with a high degree of certainty. The special staining allows highlighting the selected structure with a different color than other parts of the product. Food microscopy successfully uses a variety of staining methods. Calleja staining with green or blue Tricrom, Red alizarin can be used to detect the presence of bone fragments. Using these protocols, it is possible to monitor not only the dispersion of fat in the product, but also its formation in the layers of the outer coating, the special coloring with a Charvát modified Trichrome stain may expose other things, such as the presence of reprocessed products. (Pospiech M, et al., 2011).

The special staining used in the present study, were Masson thricrome stain, for the evidentiation of connective tissue in sausages, (fig. 7, fig. 8, fig. 9). and for the evidentiation of fungal structures in histological sections, GMS II (Grocott Methenamine Silver) staining (fig.10, fig.11, fig.12), was used.

Some studies have determined that microscopic methods are effective techniques for detecting unauthorized tissues in meat products. The organoleptic examination and the macroscopic examination, as well as the physicochemical technique, can not detect with precision the various tissue components in meat preparations such as minced meat. In this context, the microscopic examination is the only one that can detect tissue components and their position on the list of authorized and unauthorized products (Disbrey D.B. et al, 2000, Prayson BE, 2008a, Prayson BE, 2008b)

We consider that such an analysis, if properly performed and interpreted, may provide objective information as to verify the compliance of these products with the existing legislation and to ensure the composition and integrity of these foods

Conclusions

By means of routine microscopic examination, the HE technique, non-compliant structures of animal origin were identified: visceral fragments and parasitic structures, Masson's trichrome stain method allows a better highlighting of the connective tissue, and the GMS 2 technique can be used successfully for highlighting the fungal structures in meat products.

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