# Microscopic methods in food analysis

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## Abstract

The article describes couple of microscopy methods which can be used for analysis of raw materials and foodstuffs. Specifically, the article discusses microscopy as it relates to foodstuffs of animal origin, and its considerable potential, especially for studies involving meat products. There are many ways of processing and preparing samples for microscopic examinations today, paralleled by a variety of investigative techniques. These procedures include both classical methods and the methods employing state-of-the-art equipment. The article is focus to these areas: determination of food components and food quality, detection of food adulteration and risk ingredients in food (detection of animal constituents, detection of vegetable constituents) and quantification of food components which are describe in the article.

*Foodstuff, histochemistry, immunochemistry, light microscopy, image analysis*

## Introduction

The methods of microscopy, next to those of chemistry, immunochemistry and molecular biology, represent yet another, in some cases cheaper, alternative for the examination of food. The history of food microscopy dates back to 1850, when Arthur Hassall used a microscope to tell coffee from chicory. In the ensuing years, Hassal's results were elaborated upon by other authors, such as Clayton: Determination of Fat and Proteins, Wallis: Analytical Microscopy, Winton and Winton: Structure and Composition of Food, Böhm and Pleva: Microscopy of Meat and Raw Materials of Animal Origin, Aguilera: Microstructural Principles of Food Production and Analysis, Flint: Microscopy of Food, Tremlová: Histology of Food. Presently, in the Czech Republic, microscopic analysis of food is being pursued by a team of experts at the Department of Vegetable Foodstuff and Plant Production, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno. These researchers endeavor to combine the methods of microscopy with the most modern methods from other disciplines like immunohistochemistry, image analysis, and stereology.

Specifically, the article discusses microscopy as it relates to foodstuffs of animal origin, and its considerable potential, especially for studies involving meat products. There are many ways of processing and preparing samples for microscopic examinations today, paralleled by a variety of investigative techniques. These procedures include both classical methods and the methods employing state-of-the-art equipment. Microscopy (optical, light, electron or atomic) and other imaging technologies offer some of the best approaches to evaluate the structure of food due to the fact that the information is rendered in a pictorial form. That captures the general condition of the product better than a series of numbers. However, if needed, the microscopic image can be converted into numerical data that lend themselves to statistical processing. These techniques are typically accompanied by sensory evaluations of food on the part of both consumers and producers (Kaláb et al. 1995).

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By their focus, the capabilities of modern, food-oriented microscopy can be divided into several areas, which will be discussed in turn.
1. Determination of food components and food quality
2. Detection of food adulteration and risk ingredients in food
a. detection of animal constituents
b. detection of vegetable constituents
3. Quantification of food components

## 1. Determination of food components and food quality

Various imaging techniques are now available for microscopic determination of food components. Among the most widely used are light microscopy to study the microstructure, and transmission electron microscopy (TEM) as well as scanning electron microscopy (SEM) with higher magnifying (imaging) powers to study the ultrastructure of foodstuff. The practical applications of the last two methods are well covered by authors like Katsaras, who described in detail the mechanism of protein binding in meat products (Katsaras et al. 1994) as being essential for cohesion in the finished product.

The most commonly used method is light microscopy. It exists in a variety of modifications, of which the most popular is microscopy with transmitted light in the visible spectrum of light. This microscopy works with samples prepared by specific or basic staining to differentiate the individual internal structures. The basic staining is normally used to enhance all structures present in the final product, which are then identified by their morphological characteristics (shape, size and the mutual cell configuration, the presence of crystals, grains, or other elements). This was the procedure applied to meat products by the team of Czech authors Böhm and Pleva. The frequently used staining methods are hematoxylin-eosin (shades of red) (Plate II, Figure 1) or toluidine Blue (shades of blue) (Plate II, Figure 2), which can reveal most animal and vegetable components in meat product structures. It is possible to prove the presence of not only muscle and fatty tissues, but also for example the use of liver tissue in pates, salivary glands in the products made from head trimmings, natural guts as casings, or spices and other additives. A disadvantage of the basic staining is the fact that the individual components are show in varying shades of a single color (Plate II, Figures 1, 2). Also, if the original component structure of the product was disturbed during the processing, then the identification cannot be made with a requisite degree of certainty.

Special staining enables highlighting the selected structure with a different color than other parts of the product. Food microscopy successfully utilizes a variety of staining methods. The Calleja staining with Green or Blue Trichrome (Plate II, Figure 3), or by means of picrosirius (Flint and Pickering, 1984) red, is suitable to determine collagen, the main component of connective tissue. Alizarin red can be used to detect the presence of bone fragments. Sudan III, oil red, or sudan black (Plate II, Fig. 4) have been successfully applied to highlight the presence and structure of adipose tissues. Using these protocols, it is possible to monitor not only the dispersion of fat in the product, but also its formation in the sub-packing layers. Special staining with a Trichrome dye as modified by Charvát can also reveal other things, like the presence of re-processed products.

However, the products of meat-processing industries are not composed only materials of animal origin. We could not imagine today's meaty dishes without the characteristic smell and taste imparted by ingredients like spices, herbs, and lately also by extracts or chemical aromatics. Natural spices have always been thought of as having higher quality, and some meat products use them in larger pieces even now to enhance the appearance. The raw materials of plant origin are used not only to improve the taste and/or appearance parameters in the final product, but also for superior nutritional and structural qualities, such as better bonding or cohesion properties (textural characteristics) of the product.

A microscopic evidence of these raw materials can be obtained by special staining methods based mostly on polysaccharides that exist in the raw materials of plant origin in a large degree. For example, starches can be shown with the Lugol-Calleja stain (Plate III, Figure 5) and all vegetable polysaccharides with PAS-Calleja stain. However, the categorization of these additives must rely on the structure of cells or whole tissues.

One can encounter a variety of spices in meat production, in quantities measured in hundredths or tenths of percent, rarely as much as 1% (Valchař 2005). The most commonly used spices in meat production include black pepper and paprika, as well as caraway seed, marjoram, coriander, allspice, and others. Spices belong to those raw materials where microscopic examination has long served for verification of their purity and identity, along with the detection of presence and distribution in meat products. Microscopic evaluation of course presumes an adequate knowledge of morphology of the individual constituents. Analyzing the samples can be facilitated by those staining protocols that make the components of interest more visible. For example, the polysaccharide structure of cell walls, or some substances which are insoluble and therefore suitable for histochemical detection, can be stained pink by Schiff's reagent. The differentiation of spices from other raw materials of plant origin is possible on the basis of spice meshing that contains cells with stronger walls in greater measure. Proving the use of extracts obtained from natural spices, or merely chemical aromatics, relies on the finding of extract carriers (e.g. starches). The presence of these forms of spice additives can be also confirmed by the absence of natural spices in combination with a sensory test.

Aside from proving the presence or absence of individual components, microscopic examination can determine the suitability of technological processing, or identify which technological process was followed in making a certain food. Another possibility afforded by microscopic examination is an assessment of the quality and quantity of the raw materials used, and the mutual arrangement of the individual components in the product (Plate III, Figure 6) that affects its ultimate quality. It is possible to monitor the homogeneity or the distribution of the individual ingredients within meat products, such as dispersion of adipose tissue, or additives of plant origin, that is to say starches, vegetable proteins and carrageenans. The microscopic examination of food in this sense is an effective, and often irreplaceable, tool.

Quality assessment of meat products by microscopic methods can be applied to raw materials and finished products alike. In monitoring the incoming raw materials, food microscopy finds its application in tracking the changes in the mechanically separated meat by means of polarizing (Branscheid 2002) or transmitted light microscopy (Pickering et al. 1995), (Tremlová et al. 2006). Another option is to distinguish the natural and modified plant additives, evaluate their quality, purity and therefore suitability for use in the industrial processing of meat. Post-production evaluation of product quality means to determine the product structure and verify compliance with the requirements pertaining to that product category.

## 2. Detection of food adulteration and risk ingredients in food

The basic raw materials of animal origin are meat, milk, eggs and honey. The microscopic structure of all materials of animal origin has been relatively well documented in literature, down to the electron microscopy level. From the perspective of microscopy, the most intricate raw material in general is meat, being a complex of various cells and tissues. On the other hand, these structures, clearly visible under a light microscope, enable the identification of meat. However, meat is not only muscle tissue as most people would imagine, but, legally speaking, all edible parts of bodies of animals of different kinds, of mammals as well as birds, inclusive of wild animals. Therefore, meat is not only muscle tissue, but also the internal organs and blood (ES 2004). All of these components are

composed of different types of tissues whose presence is the basis for identification. It is especially the cross-striated muscle tissue, the adipose tissue, the connective tissue and other types of tissues that characterize the internal organs (glandular epithelium (Plate III, Figure 7), blood vessel configuration, etc.). The quality of meat that is present in meat products is measured especially by the content of fat and connective tissue (Decree 2001). The evaluation should take into account the changes that these tissues sustained during technological processing (tearing of muscle fibers, swelling, etc.), since the processing, particularly mechanical, could be so intense that it may greatly complicate the correct diagnosis (Tremlová 2003). Therefore, the morphological criteria applied to the individual components of meat products are used in conjunction with other techniques to create a contrasting image, be it physical (e.g. polarizing microscopy), chemical (histochemical staining protocols), or their combinations. Mechanically and thermally processed foodstuff may be successfully studied by histochemical methods, which combine histopathology and chemistry, with the objective of identifying and localizing chemical substances in the cells and tissues of a histological section. It is not only a simple matter of detecting a chemical substance, but also the knowledge how these substances are distributed and where they are deposited in food. The goal of such a determination may be to visualize proteins, nucleic acids, polysaccharides, lipids, enzymes, or inorganic substances. These chemicals then serve as markers for the presence of certain raw materials or ingredients in food. The substances with antigen properties (specific proteins) are localized by immunohistochemical methods, for example when checking the constituents of muscle tissue such as actin, myosin or collagen. It is possible to show the brain tissue immunohistochemically, even in the thermally processed meat products (Tersteeg et al. 2002). These special microscopic methods could be deployed to identify milk-based or egg-based raw materials (e.g. whey protein) within meat products.

**a. Detection of animal constituents**
   A standard microscopic examination in meat products can detect different types of muscle tissue, the individual types of connective tissue, adipose tissue, bone fragments and parts of organs (Plate III, Figure 8), all of which can be used to assess a potential adulteration of food. The diagnostic work is usually made more difficult by a thorough mechanical processing of the materials. The identification of the individual tissues is facilitated by special staining. The content of collagen and adipose tissue is partially indicative of the quality of the meat used in production (Plate IV, Figure 9). The incorporation of mechanically separated meat where not allowed or not declared is also considered an adulteration. The proof is usually based on a chemical detection of bone tissue (calcium ion determination). In a histological section, the bone tissue can be well highlighted and there are several protocols for special staining to do that, such as Alizarin Red or staining by Kossy (Plate IV, Figure 10). Bone fragments do not normally occur in meat products, so their presence in these exceptional cases may indicate the problem of a poorly processed raw material, although more often it is the inclusion of mechanically separated meat. The method can even be used to assess how standard is this material, whose properties depend on the sourcing, the machine adjustment, and the nature of the raw material used.
   It is also possible to recognize, microscopically, the reworked products (usually non-standard) into the new ones. The conclusion is based on the sub-package layer of the original product having a different character and discoloration than the inner parts (due to heat processing or smoking), and it is often possible to detect even the packaging of the original product. The examination can be helped by a special Trichrome stain (Plate IV, Figure 11), but an experienced investigator recognizes a re-processed item even with the standard Hematoxylin-Eozin stain.

Problematic is the detection of processed skin, which is referred to as "skin emulsion" in practice. The processed skin is a material containing primarily a large quantity of stromatic proteins (mainly collagen) which thermal processing converts into gelatin, thereby affecting the structure in the finished product. Products with larger amounts of processed skin typically stand out under the microscope by having a smaller portion of muscle tissue relative to the macerated portion (Plate IV, Figure 12).

### b. Detection of vegetable constituents

A normal microscopic staining can easily identify the constituents of plant origin, especially in their traditional form such as flours and starches. The proof of their presence may be based on certain structural elements (e.g. aleurone cells of cereal seed coat or palisade cells of soya), or on the evidence of typical constituents (e.g. reserve protein). Specifically, the addition of flour and starches is easily provable, particularly with special staining showing starch (or possibly polysaccharides). It is possible to use the Lugol-Calleja stain (Plate V, Figure 13), which colors the grains of starch dark-purple to black, or the PAS-Calleja stain (Plate V, Figure 14), which colors all the polysaccharides pink. This staining is also suitable for a microscopic proof of fibrous material, which, in a microscopic slide, appears as disordered, solidly outlined fibers without any particular shape. Among the fiber-rich materials used as additives in meat products are especially potatoes and wheat.

When using the basic staining, vegetable additives may be confirmed only on the basis of a typical appearance and structure in the microscopic image (Plate V, Figure 15). Wheat flour contained in a product forms clusters containing grains of starch altered by the heat treatment. Wheat starch, and other common starches, can be recognized by their typical shape (see Table 1). In a microscopic examination, some of the starch grains also show a typical core-centered layering, created by air-filled tears of various shapes. This characteristic microscopic image, however, may be altered by technological processes (heat treatment, starch modification, etc.). The modified starches in microscopic slides, depending on the treatment, appear as fibrous formations with a partially preserved circular shape. Their identification relies especially on a proof by a color change achieved by special staining (Lugol's solution).

Table 1. Microscopic description of selected grains of starch

| Starch | microscopic description of starch grains |
| --- | --- |
| Wheat starch | Small and large grains shaped like lentils with tiny central layering |
| Potato starch | Egg-shaped or elliptic grains with an eccentrically located core and off-centre layering |
| Corn starch | Multi-faceted, tile-shaped grains with a star-like core |

Horn (1987) describe that microscopic examination of meat products enables to prove the inclusion of vegetable protein additives in the products, if they occur in sizes suitable for light microscopy. Vegetable proteins, depending on the type, come in the characteristic shapes of sponges, sickles or rings accompanied by small remnants of saccharides (Flint 1994). Wheat protein has been likened to a sponge formation with holes (Plate V, Figure 15). The microscopic determination of soy protein can also rely on its morphological structure that has been described as spongy, sickle-shaped, or ring-shaped according to the type of protein (Plate V, Figure 16). The commonly used staining protocols cannot clearly differentiate pure vegetable protein from other proteins in the product (Tremlová and Štarha 2002). Only when using basic staining with Toluidine Blue, the vegetable proteins become different shades of blue (wheat protein: light cyan, soy protein: dark blue) in contrast to other components within the product. In the case of soybean flour or textured soy protein, or possibly some soy concentrates, the proof also involves structural elements,

such as palisade cells and cup cells, and, likewise the wheat flour, with a visible layer of aleurone cells. This examination can be facilitated by the previously mentioned targeted staining for polysaccharides. Using the histochemical staining methods, it is possible, as already noted, to prove the presence of wheat flour (and, indirectly, of wheat proteins) through starch coloration. Due to a high starch content (up to 80%), flour identification is not difficult, but proving the source of starch (after technological processing), possibly in the presence of wheat protein, remains, in many cases, problematic. The application of imunohistochemical methods yields a more sensitive response. Vegetable proteins can then be identified on the basis of a more prominent color difference, where the protein is highlighted in brown due to the bonding of specific marked antibodies and DAB by chromogen (Plate VI, Figure 17) against a background (blue or green) (Lukášková et al. 2008). When this color system is combined with another, e.g. with the BCIP/NBT chromogen, then the method of multiple labeling allows to check for the presence of two proteins in one test, which makes the product evaluation faster and cheaper (Řezáčová Lukášková et al. 2010). However, the immunohistochemical method has limitations when it comes to investigating wheat flour addition, which exhibit only a slight steined triggered by the binding of antibodies (Plate VI Figure 18). This phenomenon is caused by a relatively low concentrations of wheat protein (7-13%), in wheat flour, when the individual protein epitopes with bonded antibodies are too far apart and do not provide the eye with a recognizable color image (Řezáčová, Lukášková et al. 2011). In the case of soy protein, however, the amount of protein in all types of additives (soy flour, concentrate, isolate, texturate) is sufficient for a reliable immunohistochemical proof (Pospiech et al. 2009).

## 3. Quantification of food components

As already mentioned, the microscopic methods can be used to examine different kinds of meat products in terms of both raw materials and technology. In practice, the vast majority of cases are a matter of qualitative evaluation performed to verify the presence or absence of a certain component. However, these methods can be expanded to include a quantitative examination of food by means of image analysis. Scientific literature considers the results obtained during the examination of meat and meat products by image analysis to be objective, accurate and comparable to the data obtained by chemical methods. The suitability of this method is not limited to the category of food (determination of the sensory, technological, and qualitative value in various food commodities), but is also applicable to medicine, criminal justice, and chemical industries. The method enables to describe and specify quantitatively the images information obtained from macroscopic or microscopic images. The resultant numerical data from the image make it possible to compare different samples in minute detail, process the generated information accurately, and express the results in statistical terms. A great advantage is also the ability to compare the samples under examination with the ones previously examined within the system (Brosnan and Sun 2004).

A very important step for successful automatic image analysis is the correct selection of special staining for the monitored component. The most suitable staining for the determination of fat turns out to be Oil Red (Plate VI, Figure 19). Green Trichrome is suitable for collagen determination (Plate VI, Figure 20), while bone fragments may be identified with the aid of Alizarin Red (Plate VII, Figure 21). The best staining to quantify an additive of plant origin, such as starch, is Lugol-Calleja (Plate VII, Figure 22), because this particular histochemical stain provides a sufficient contrast between the grains of starch and the other components in the product.

The image analysis consists essentially of taking photographs and having them analyzed by a computer program. A light microscope and a digital camera can be set up to scan histological sections. The actual analysis involves the creation of a template (from the

image parameters, usually color and brightness) to identify the selected components, and the subsequent measurement of their areas and the section area. The acquired data can then be processed statistically. This method is suitable for analyzing other types of foods as well. With cheeses, the method can be used for example to evaluate the morphology of fat crystals, which affect important properties such as spreadability, hardness, and fineness in the finished product. In the area of fruits and vegetables, it is widely used to control robotic manipulators or vehicles for rapid and effective harvesting. Another possibility to use image analysis is for on-line measurements, which is very beneficial, or even necessary, in maintaining quality control in food manufacturing. The main advantage is the ability to get results without a direct contact with the sample. This greatly minimizes the danger of cross-contamination and similar risks (ES 2004).

Currently, the meat processing industry uses image analysis to evaluate meat and meat products, as a method that yields objective and accurate results comparable to those obtained by chemical methods. This includes, for example, the use of image analysis to objectively determine the marbling of meat, or to measure the various muscle fiber parameters that are useful in monitoring the quality of meat (Buche and Mauron 1997). Image analysis can also be used to quantify the amount of tissues such as collagen-type, elastic connective tissues and bones, especially in mechanically separated meat, as an indicator of product quality (Albrecht et al. 1996).

Image analysis of meat products can also be used to detect vegetable components. This approach turned out to be very useful in identifying the vegetable protein additives in meat products, for example those in the category of patés (Boutten et al. 1999). At present, several protocols are available to demonstrate the presence of vegetable proteins. These methods are mostly immunochemical. Brehmer et al. (1999) used the ELISA method for a quantitative proof of several kinds of vegetable proteins (such as soy or peas) and of gluten in heat-processed and non-heat-treated meat products. A quantitative determination can be also performed by a combination of microscopic and immunological methods (e.g. immunohistochemistry), and the method of computerized image analysis (Randulová et al. 2011).

## Conclusions

Microscopic methods can determine the individual components of food and evaluate their presence with regard to the possibility of food adulteration. Unquestionably, the microscopic methods have a considerable potential and importance for food examinations, especially when the classic investigative techniques are empowered by with modern evaluative procedures. They meet the requirements of speedy results and food component specificity. A number of such methods have been qualified in our laboratory and they are ready for to be deployed in practice. The methods for microscopic examination of foods from this project are published at www.foodmicroscopy.com website, which will be periodically updated. The microscopic methods coupled with image analysis also open the possibilities of quantifying the results and processing them statistically. Significant is also the ability to analyze the different image parameters for a comprehensive evaluation of the sensory and technical properties of foods and their raw materials.

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Plate II
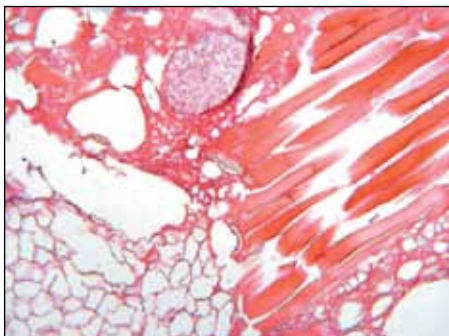Pospiech M. et al.: Microscopic methods ... pp. 27-34



Fig. 1. Basic staining: Hematoxylin – eosin, the structure of meat products 100×
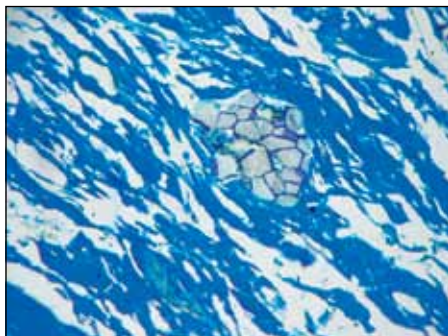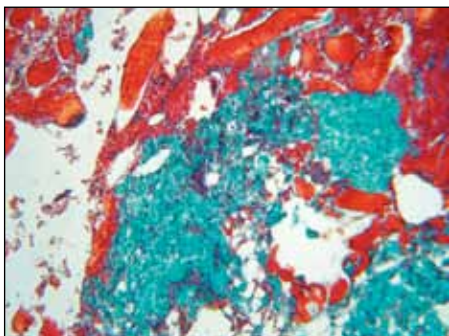


Fig 2. Basic staining: Toluidin blue, 100×



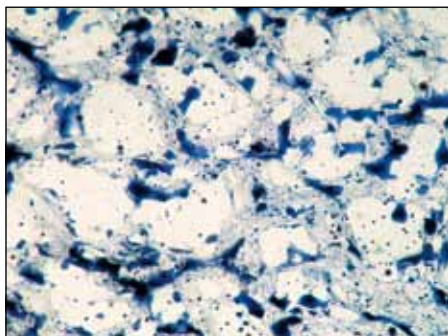Fig 3. Special staining: Green trichrom, collagen - green, 100×



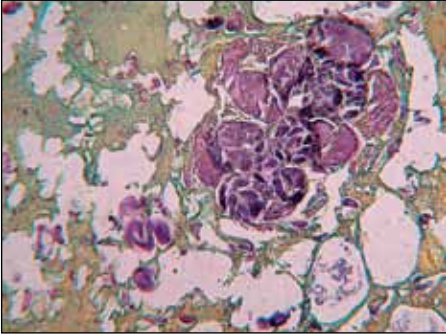Fig 4. Special staining: Sudan black, dispersion of fat in meat products, 100×

Plate III



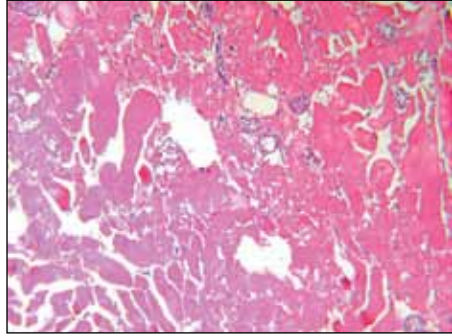Fig 5. Special staining: Lugol Calleja, starch grain - brawn, 100×



Fig 6. Basic staining: Hematoxylin – eosin, distribution of soya proteins in meat products 100×
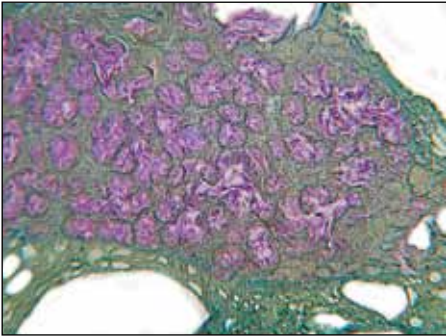


Fig 7. Special staining: PAS Calleja, detection of salivary gland - pink, 100x
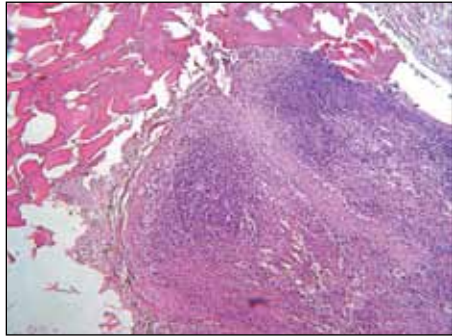


Fig. 8. Basic staining: Hematoxylin – eosin, limph node– blue, 100x

Plate IV
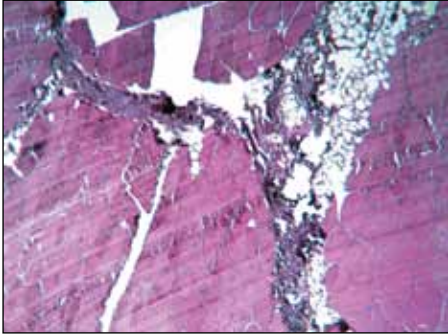


Fig. 9. Basic staining: heidenhains azan, ratio of muscle and fat, 250x
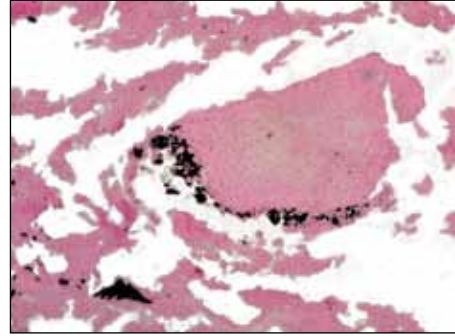


Fig 10. Special staining: staining by Kossa,calcium - black, 100x
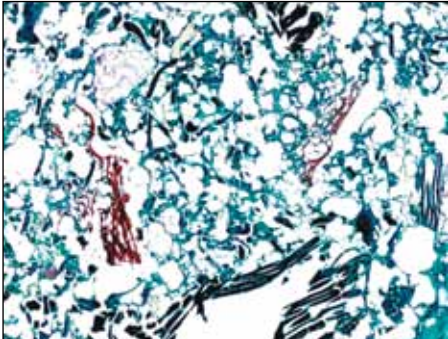


Fig 11. Special staining: Blue trichrome modified by Charvát, reworked products - red, 100x, sources T. Grunewald
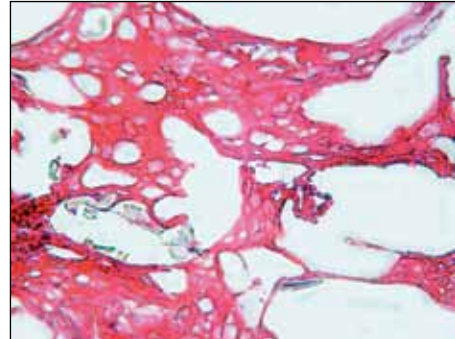


Fig 12. Basic staining: Hematoxylin – eosin, meat products with processed skin, 400x

Plate V
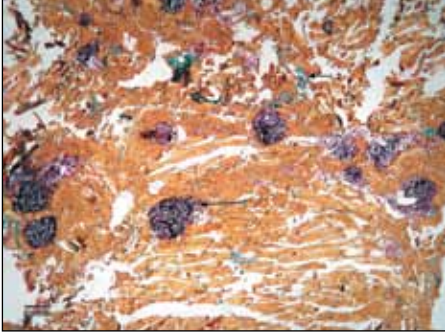


Fig. 13. Special staining: Lugol Calleja, wheat flour – black, 100×
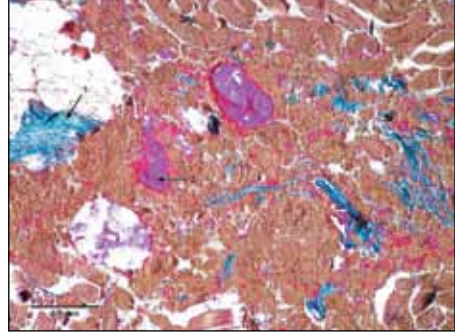


Fig. 14. Special staining: PAS Calleja, wheat flour – pink, 100×
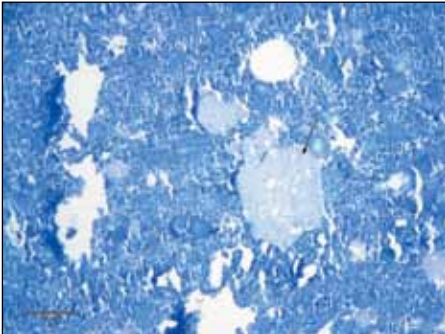


Fig. 15. Basic staining: Toulidin blue, azure blue – wheat proteins, 100×
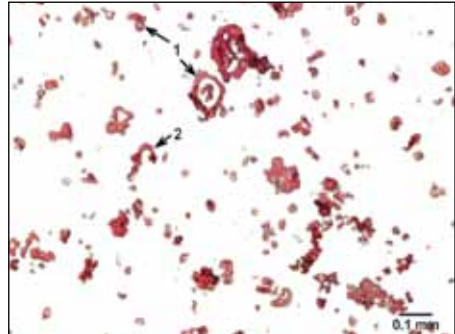


Fig. 16. Basic staining: Hematoxilin – eosin, soya protein, 400×

Plate VI
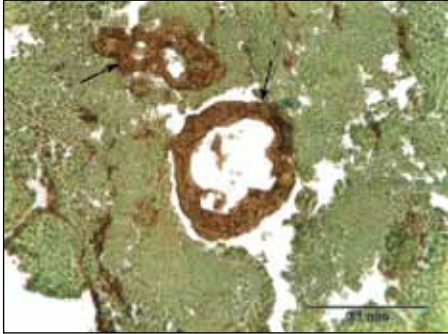


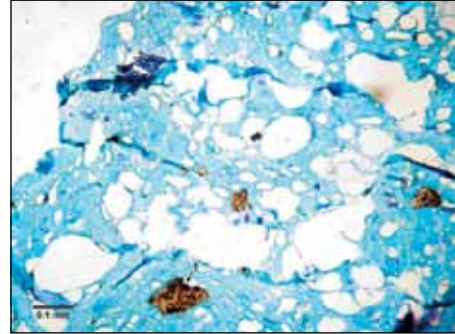Fig. 17. Immunohistochemical staining, soya protein – brown, 400×



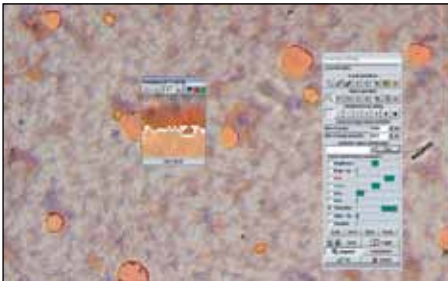Fig. 18. Immunohistochemical staining, wheat protein – brown, 100×
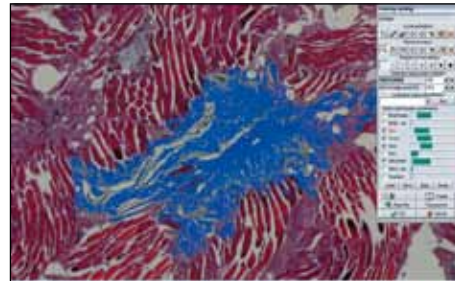


Fig. 19. Image analysis of fat, Oil red



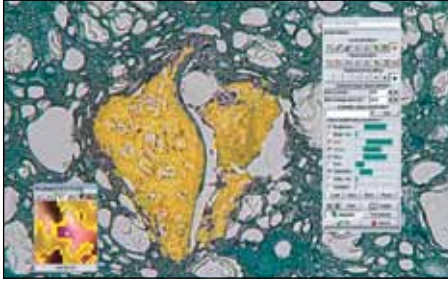Fig. 20. Image analysis of collagen, Green trichrom
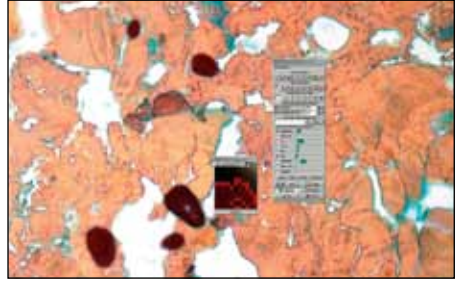
Plate VII





Fig. 21. Image analysis of bone fragments, Alizarin red

Fig. 22. Image analysis of starch, Lugol Calleja