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POSSIBILITIES OF MICROSCOPIC DETECTION OF ISOLATED PORCINE PROTEINS IN MODEL MEAT PRODUCTS

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ABSTRACT

In recent years, various protein additives intended for manufacture of meat products have increasing importance in the food industry. These ingredients include both, plant-origin as well as animal-origin proteins. Among animal proteins, blood plasma, milk protein or collagen are used most commonly. Collagen is obtained from pork, beef, and poultry or fish skin. Collagen does not contain all the essential amino acids, thus it is not a full protein in terms of essential amino acids supply for one's organism. However, it is rather rich in amino acids of glycine, hydroxyproline and proline which are almost absent in other proteins and their synthesis is very energy intensive. Collagen, which is added to the soft and small meat products in the form of isolated porcine protein, significantly affects the organoleptic properties of these products. This work focused on detection of isolated porcine protein in model meat products where detection of isolated porcine protein was verified by histological staining and light microscopy. Seven model meat products from poultry meat and 7 model meat products from beef and pork in the ratio of 1:1, which contained 2.5% concentration of various commercially produced isolated porcine proteins, were examined. These model meat products were histologically processed by means of cryosections and stained with hematoxylin-eosin staining, toluidine blue staining and Calleja. For the validation phase, Calleja was utilized. To determine the sensitivity and specificity, five model meat products containing the addition of isolated porcine protein and five model meat products free of it were used. The sensitivity was determined for isolated porcine protein at 1.00 and specificity was determined at 1.00. The detection limit of the method was at the level of 0.001% addition. Repeatability of the method was carried out using products with addition as well as without addition of isolated porcine protein and detection was repeated 10 times. Repeatability in both, positive and negative samples, for isolated porcine protein was determined at 100%. The results show that the histological processing of cryosections stained using Calleja is suitable for detecting isolated porcine protein in meat products.

Keywords: meat; collagen; isolated protein; light microscopy

INTRODUCTION

In recent years, development of a variety of functional ingredients for the most possible efficient optimization of the quality and texture of meat products is increasing in the meat industry. Functional raw-materials of animal origin include different animal derivatives of meat, skin and blood, as well as milk and egg products (collagen, gelatin, whey protein, casein, albumin, dehydrated beef protein, and other protein isolates) **(Petracci et al., 2013)**. Proteins play an important role in the functional and sensory properties in meat products. Vegetable proteins often give the product an atypical flavor, reduce the meaty taste, and many are representatives of allergens. Therefore, animal protein isolates as functional ingredients in meat industry are rising again **(Khiari et al., 2014)**. The basic reasons to use protein additives include: increasing nutritional value of meat products, improving technological properties of processed raw materials, improving sensory characteristics of finished products, and last but not least, there are also economic reasons **(Lat et al., 1984)**. In their study, **Prabhu (2004)** reports that isolated porcine protein containing 85% protein and 12% fat obtained from fresh pork trimmings by a technology at low temperature is able to attach as much water as up to four times its weight.

The nutritional value of proteins is then judged mainly according to the amino acids contained, in particular by the essential amino acids contained. Nutritional value of animal proteins is high, while value of plant proteins is considerably lower because they lack some essential amino acid (AAs) (lysine or sulfur-free AAs). Proteinaceous ingredients are produced with a protein content of 50 – 90% **(Straka and Malota, 2006)**. The most natural alternative to meat proteins are therefore animal proteins which are widely used in meat production. For some types of meat products, addition of animal proteins is necessary, e.g. gelatin applied in cooked meat products and specialties. The most common protein used in the production of ground meat products are blood proteins, milk proteins, egg proteins, and collagen proteins **(Budig and Mathauser, 2007)**. Collagen is a group of insoluble fibrous proteins found in all multicellular organisms. It is the most abundant protein in mammals,

amounting approximately to 25 – 30% of the total body protein. Collagen is a major component of skin, bones, cartilages, tendons, blood vessels, basement membranes and teeth **(Tarté, 2010)**. Native collagen consists of alpha 1 and alpha 2 chains, which differ in their order of amino acids. These chains form a triple helix called tropocollagen. It is approximately 256 nm long and it represents the basic unit of collagen. Secondary structure of collagen is then formed by a left-handed helix of an elongated type with thread pitch of 0.95 nm and individual peptides in 0.286 nm distance from each other. The tertiary structure of collagen consists of three chains that are intertwined and have a central axis. The resulting rod-shaped configuration is about 290 nm long with a diameter of 1.4 nm and it is called tropocollagen **(Khoshnoodi et al., 2006)**. For meat industry, collagen sources include skin, bones, entrails and skeletal muscle **(Bailey and Light, 1989)**. Collagen may be added to meat products as an ingredient (meat raw materials rich in collagen: skeletal muscle with connective tissue, pork skin) or in concentrated form as a direct additive. This can be produced from bones (bone collagen extract), pork skin, and skeletal muscle with connective tissue **(Gillett, 1987)**. Collagen of skeletal muscle can be concentrated mechanically or extracted by low temperature rendering followed by extrusion, dehydration, grinding, flaking, milling **(Gillett, 1987)**. These forms of collagen can significantly affect the processability and sensory properties of meat products.

MATERIAL AND METHODOLOGY

Manufacture of Model Meat Products

Model meat products were manufactured with the addition of various types of isolated porcine proteins. The used proteins included blood protein: pork plasma P; collagen-type proteins: PF, pork gel, Scancure DI 100, Scancure 95; combination of collagen-type proteins and pure muscle proteins: Scanpork D 80, Scanpork D 90 (Scanflavour, Denmark). The first set of model products was made of poultry meat with additions of 7 types of various isolated porcine proteins at concentration of 2.5%. The second set of model products was made of pork and beef in the ratio 1:1 with additions of 7 types of various proteins at concentration of 2.5%. Other ingredients to all model products included salt at a final concentration of 1.5% and 10 mL of water. Model samples were ground in a blender, then shaped and pressed in a ham mould in which they were boiled for 10 minutes after reaching 70 °C in its core, subsequently they were cooled down, cut into cubes and placed in a freezer. Next, cryosections were cut at the HM 550 cryostat (Germany, Microm). The sample was attached to a metal stip of the freezing microtome using Killik freezing medium. The stip was left in the freezer bar of the cryostat to solidify. The sample was thus ready for cutting, so it was attached to the holder and slicing started. 16 μm thick sections were transferred to specific Thermo Superfrost slides (Germany, Thermo scientific). 42 sections on 21 slides were made from each sample – each slide contained two sections. After adhering, the sections were stored at a cooling temperature until further processing.

Histological Staining

The sections were stained using differential and targeted staining. For each staining 3 slides containing 2 sections each were used. From differential stainings, hematoxylin-eosin and toluidine blue staining and targeted Calleja were applied. The prepared samples were examined using Leica DM 3000 microscope (Germany, Leica). Images were created using Leica DFC 295 camera (Germany, Leica) connected to a computer, by means of Xn Wiew software. Based on the results of this part of the experiment, the most suitable method to detect isolated porcine proteins was selected and pork protein called Scancure 95 by Scanflavour (Denmark) was chosen to be used for the production of model meat products in the second phase of the work, which was aimed at determining the detection limit of this method.

Samples for Determination of the Detection Limit

The following ingredients were selected for the second experiment: pork and beef in the ratio of 1:1 and isolated pork protein called Scancure 95 by Scanflavour. The following concentrations of samples were produced: 0.001%; 0.01%; 0.10%; 1.00%; 2.50%; 5.00%. Other ingredients to all model products included salt at a final concentration of 1.5% and 10 mL of water. Model samples were processed according to the procedure in the previous chapter. And they were stained by targeted Calleja.

RESULTS AND DISCUSSION

In the first step of the protocols, model meat products were manufactured from available commercially produced isolated porcine proteins. A combination of poultry, pork and beef meat was used as the basis of individual meat products in order to find the most suitable combination for the production of model meat products. Model meat products were manufactured with the addition of isolated porcine protein in concentration of 2.5%. This step resulted in the selection of the best detectable isolated porcine protein. Based on processing and examining samples of poultry meat and the combination of pork and beef, it was found that the raw material itself has no effect on the microscopic detection of isolated porcine protein. The raw material in particular affects processing of the samples and primarily it's cutting and staining. For this reason, the combination of pork and beef was used in the next phase.

The most widely used collagen protein (in terms of production of ground meat products) is collagen protein powder of pork origin obtained from pig skins. These are used primarily where there is a need to increase elasticity, improve slicability and cohesion. Stabilization of the product is achieved by a combination of collagen protein and other proteins of animal origin, e.g. blood plasma. Another property is a significant contribution to the reduction of syneresis in the finished meat products packaged in a vacuum or a modified atmosphere. Pork skins in the form of skin emulsion are used most commonly **(Budig and Mathauser, 2007)**. Functional properties of the added collagen used in meat products depend on the species and age of animal, anatomical sources, and extraction conditions. The potential use of collagen as an additive in meat products dates back to

approximately 1960 **(Tarté, 2010)**. From the created staining range applicable to detect collagen in practice, it was necessary to determine which ones are suitable to identify isolated porcine protein. They were verified by 2 differential and one targeted staining. From among differential staining, haematoxylin - eosin and toluidine blue stainings were used and, from among the targeted ones, Calleja was used (Figure 1). Targeted staining procedures were focused on the detection of collagen which should be distinguished from other structures in the product in each staining. All stainings applied are primarily intended for paraffin sections. However, our experiment showed that they are also suitable for cryosections. Cryosections were used because their examination is financially less demanding as well as less time consuming.

The first differential staining was hematoxylin - eosin. This staining is not too demanding on chemicals and time, but it does not belong among the simplest ones. It is highly effective in distinguishing, all the structures in the sample can be identified, even though they are only in various shades of red and blue. Isolated porcine protein is dark pink, sometimes up to crimson. An experienced examiner can recognize it because of its characteristic structure. Collagen protein has a net-like structure with cores forming different circular shapes in the margins. No sections were lost in this process due to floating away.

The second differential staining was toluidine blue. This method is neither demanding in terms of chemicals nor of time. Of all the applied stainings, this one is the easiest and quickest. It is very effective in terms of substances identification. Collagen protein is slightly purple and it is characterized by its typical structure. No cryosections were lost.

The targeted staining was performed according to Calleja. Calleja staining is relatively time-efficient. It is very effective in differentiation. There is a clear distinction in structures within the meat product. Collagen protein is stained kerosene or bluish-green and it is clearly distinguishable from other structures that are predominantly green (Figure 2). Another advantage is limited losses of sections. For these reasons, this staining method was selected as the most suitable for the detection of isolated porcine protein in meat products.

Method Validation

A graded series was produced in order to determine the detection limit. A combination of pork and beef in the ratio of 1:1 and isolated pork protein of Scancure 95 (Scanflavour, Denmark) were utilized. This isolated pork protein has a high content of collagen protein in its composition and thus it was the most suitable material for the microscopic detection. Scancure 95 is composed of 98% collagen protein and 1 – 2% pure muscle proteins. It is intended as an addition to smoked meat. The following concentrations were created: 0.001%; 0.01%; 0.10%; 1.00%; 2.5%; 5%. Of the above staining methods, Calleja was selected as the most suitable for the detection of isolated porcine protein because of the best color contrast between different structures in the meat product. Therefore, the following parameters were also established for this staining in order to validate this method: sensitivity and specificity, and repeatability. The method was validated as a qualitative method, i.e. a method to detect whether the analyte is present or absent. These methods are common in routine laboratory testing in particular as the first step for subsequent determination of the concentration of the investigated substance. They can therefore be ranked among screening methods. The advantage of its use is the reduction of costs as well as time **(Trullols et al., 2004)**. As with quantitative methods, user must be confident that the results of the qualitative method applied are suitable for the aimed purposes, which means that the method must be validated **(European Commission, 2002)**. Commonly selected methods for validation are based on quantitative methods, and there are also many validation procedures which are accepted by the supervisory authorities and professional community working in the field **(Trullols et al., 2004)**.

For detection of isolated porcine protein, the detection limit was determined at 0.001 % addition. Using isolated porcine proteins in meat products can be expected at about 10 % as suggested by a study **(Doerscher, et al., 2003)**. With regard to the optimum concentration applicable in meat production, the method is suitable for detection of isolated porcine proteins in meat products.

The evaluation criteria for qualitative methods inlcude also sensitivity and specificity **(European Food Safety Authority, 2004)**. For qualitative methods, sensitivity means the ability of a method to detect truly positive

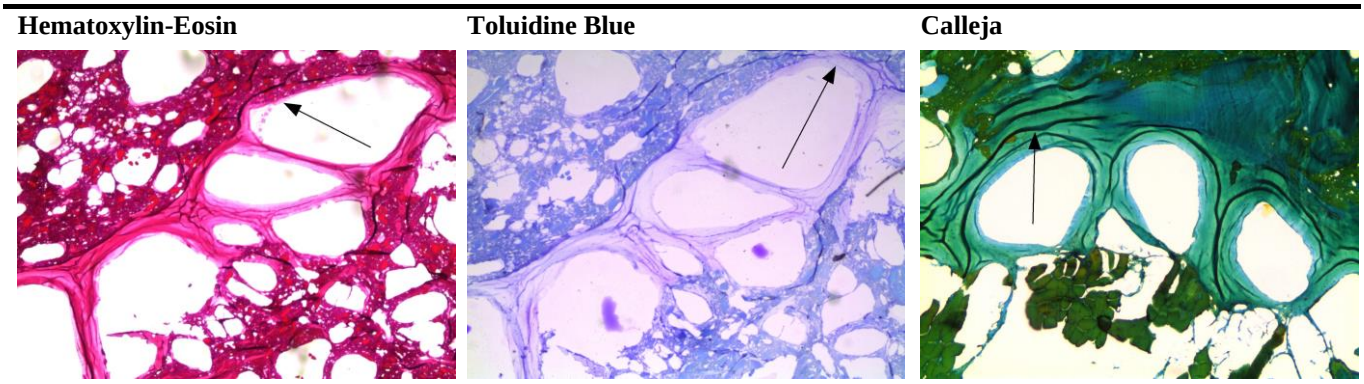

Figure 1 Isolated porcine protein in a model meat product (25 x magnification).

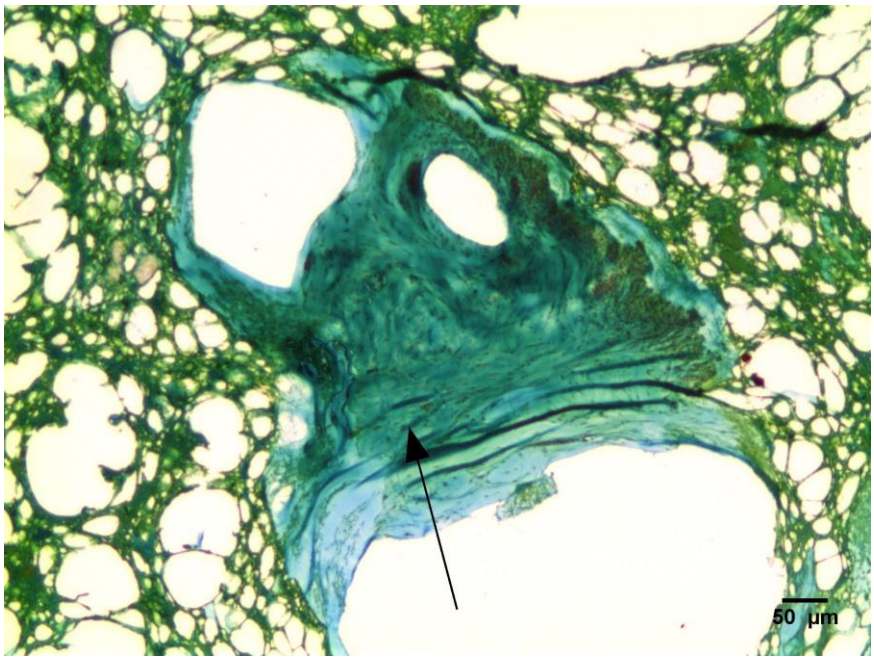

Figure 2 Isolated porcine protein (bluish green) in a model meat product (25 x magnification).

Table 1 Detection of sensitivity and specificity of the Calleja method.

Sample no.	1/2	1/3	1/4	1/5	1/6	2/2	2/3	2/4	2/5	2/6
Declaration	N	N	N	N	N	P	P	P	P	P
Examiner A	N	N	N	N	N	P	P	P	P	P
Examiner B	N	N	N	N	N	P	P	P	P	P

Note: Declaration – N (negative), P (positive)-addition of the isolated porcine protein in model sample.
Sample no: 1/2- 1/6: model sample without the isolated porcine protein.
Sample no: 2/2- 2/6: model sample with the isolated porcine protein.

samples as positive **(O'Rangers and Condon, 2000)**. The sensitivity rate is thus the probability for a given concentration, that the method will classify the test sample as positive **(Massart et al., 1997)**. In contrast, specificity is defined as the ability of a method to detect truly negative samples as negative **(O'Rangers and Condon, 2000)**. The specificity rate is thus the probability, for a given concentration, that the method will classify the test sample as negative **(Massart et al., 1997)**. For Calleja staining method, sensitivity was determined at 1.00 for isolated pork protein. Specificity was determined at 1.00 according to the protocols **(Trullols et al., 2004)** (Table 1). An optional parameter for qualitative methods is also method repeatability. For histological methods, this parameter is particularly suitable because cutting the samples, sample processing and staining can take place on different days. This parameter is recommended to exclude the role of the environment. Repeatability of the method was carried out using the product with addition as well as without addition of isolated porcine protein and it was performed in harmony with the protocols **(Suchánek, 1999)**. Evaluation was performed by two examiners and the measurement was repeated 10 times. There was a 100% match.

CONCLUSION

Besides dairy products and bread, meat and meat products belong to the basic foodstuffs needed for good human nutrition. Above all, however, they are consumed due to their organoleptic properties. Nevertheless, with regard to the pressure on meat products buying prices and special offers in retail chains, industrial manufacturers have to substitute a technologically substantial portion of binding meat either by cheaper meat raw materials of inferior quality or by suitable ingredients and additives in order to stabilize the product, which applies in particular to ground meat products. To achieve the desired properties in the finished meat product, manufacturers use a considerably cheaper source, namely collagen proteins in the form of isolated porcine proteins. Due to their very specific functional properties, collagen proteins are used in particular for products where it is necessary to increase their elasticity, improve slicability and cohesion. Application of collagen protein significantly contributes to the reduction of syneresis in finished meat products

packaged in a vacuum or a modified atmosphere. In combination with other animal proteins, such as blood plasma, collagen proteins are also used as ingredients that facilitate emulsification or stabilization of the ground meat products. The aim of this work was to detect isolated pork protein in model meat products using histological staining of cryosections and light microscopy. In the first part of this work, 7 model meat products from poultry meat and 7 model meat products from beef and pork in the ratio of 1:1, which contained 2.5% concentration of various commercially produced isolated porcine proteins, were examined. These model meat products were histologically processed by means of cryosections and stained with hematoxylin-eosin staining, toluidine blue staining and Calleja staining. The second part focused on validation of Calleja method, where model meat products with the addition of isolated porcine protein of Scancure 95 containing 98% of collagen protein and 1 – 2% of pure muscle protein were used. The sensitivity was determined for isolated porcine protein at 1.00 and specificity was determined at 1.00. The detection limit of the method was determined at 0.001% addition. Repeatability in both, positive and negative samples, for isolated porcine protein was determined at 100%. The results show that the histological processing of cryosections stained using Calleja is suitable for detecting isolated porcine protein in meat products.

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