RELIABILITY OF DIFFERENT HISTOLOGICAL METHODS FOR ESTIMATION OF MUSCLE FIBER STRUCTURE IN MSM

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Abstract – Histochemical methods based on either well-established Toluidine Blue staining or contemporary immunohistochemical labeling based on myosin and laminin antibodies were applied on comminuted chicken meat to evaluate the potential of different histological methods to objectively detect muscle tissue and muscle tissue degradation. Also, Haematoxylin-Eosin staining was studied for the same purposes. Since the ultimate goal is to develop automated image analysis software, the prerequisites of image capturing were of interest as well. All investigated methods made the visualization of muscle tissue possible, but classification of samples of different degrees of muscle degradation into defined categories varied. Taking following aspects, such as image contrast and quality, into consideration, the immunohistochemical method with myosin and laminin antibodies, which in addition has an advantage of exploiting fully automated imaging equipment, provides objective images with good representativeness to determine the content of muscle tissue and evaluate the level of degradation in comminuted chicken meat samples.

Keywords – haematoxylin-eosin, laminin, MSM, myosin, toluidine blue

I. INTRODUCTION

Mechanically separated meat (MSM) technology is a method to improve yield and sustainability of meat production. Throughout the years, development of the technology for mechanical processes of meat-bone separation has made it possible to produce raw meat material that has good quality, and could not be distinguished from regular minced meat. However, MSM is generally considered of inferior quality, and is subjected to strict regulations, while manually separated meat is accepted as regular meat, and is not compelled to same restrictions. This differentiation is made only based on the production process, but there are no objective histological techniques that classify all MSM as being of inferior quality. Therefore, the present work focuses on finding a procedure to evaluate the potential of different histological methods to detect muscle tissue and muscle tissue degradation on mechanically separated chicken meat. Three histochemical methods (German, UK, and Danish), were applied to study the potential and outcome of the methods relative to one another.

The German and UK methods are both based on Toluidine Blue (TB) staining, which is a conventional and widely used method for staining biological material. TB has high affinity for acidic tissue components and nucleic acids, and TB stained tissues appear royal blue. However, since TB is also a metachromatic dye, structures containing glycosaminoglycans, such as cartilage, mucin and mast cell granules, may appear purple [1]. In the German method, the histological assessment of muscle fiber structure of MSM produced under various conditions of low and high pressure recovering systems uses a 4-point scale from 1, as excellent structure, to 4, as loss of structure, according to a slightly modified method by Branscheid et al. [2]; while in the UK method, developed by Leatherhead Food Research Association (LFR), Department for Environment, Food and Rural Affairs (DEFRA), and Food Standards Agency (FSA), the focus is primarily on the loss or modification of muscle fiber structure, but also other structural aspects, such as dispersed protein and connective tissue, are considered. In addition, a subjective numerical assessment of muscle fiber damage has been introduced, so that comparison between the methods and an image analysis system could be made. The alternative Haematoxylin-Eosin (HE) staining method was

conducted in order to obtain additional information for more precise estimation of muscle tissue. HE staining performs well with a broad range of cytoplasmic, nuclear, and extracellular matrix features. Haematoxylin has a deep blue-purple color and stains nucleic acids, while eosin is pink and stains proteins non-specifically [3].

The Danish method based on immunohisto-chemistry uses two different antibodies: myosin (mAb clone MF-20) and laminin (mAb clone 31, 31-2), and a fully automated image analysis evaluation procedure. Monoclonal antibody MF-20 binds to myosin heavy chain of striated muscle and to light meromyosin [4], while laminin is a membranous antibody staining the striated muscle fibers, but could also show reactions with the basal lamina of blood vessels and capillaries, and nerves [5]. Laminin could also offer enhanced differentiation between damaged and non-damaged muscle [6].

II. MATERIALS AND METHODS

Materials

There were two reference comminuted meat samples composed of 11 mixtures of varying levels of minced and emulsified meat from either thigh muscle (deboned thighs) or breast muscle without the inner fillet. All samples were delivered as frozen, so prior to use they were thawed in a cooling chamber at 4 °C and then agitated to ensure homogeneity.

The German method

Material (25 g) was mixed with mounting medium (1.5 g) (Tissue tek, Leica, Germany), and then divided into 10 aluminum foil cups, which were then frozen in liquid nitrogen-cooled isopentane, and stored at -20 °C until further use. The samples were cut in a cryostat (Leica CM3050, Germany) at -20 °C as 12 µm thick sections, collected on glass microscope slides (two sections per slide), and kept at room temperature. TB staining was done according to Branscheid et al. [2] with 0.03% solution, HE staining with 0.6% haematoxylin solution and 0.1% eosin solution. The coverslips were fixed with water-insoluble histofluid (Eukitt). Microscopic work was conducted on a Leitz Wetzlar transmitted light microscope equipped with a digital color CCD camera (x0.5: 1/2) (Leica EC3, Germany). The objective was PL APO 16x, NA 0.40, and image resolution 2048x1536. Three images per slide were acquired, and the obtained images were graded according to the method, and an estimate on % structured muscle was given (Table 1).

Table 1. Grades of MSM according to the German method, and correspondence to % of structured muscle.

Grade	Description	% structured muscle
1	Completely free or slight modifications of single fibers without affecting the structural grid	100-75
2	Slight traces or clear traces of grinded fibers	74-50
3	Grinded fibers or massively grinded fibers	49-35
4	Complete disintegration of the structure, squeezed-out matrix of cartilage	34-0

The UK method

Small amounts of samples were placed on a metal stubs using Tissue tek as a mountant, and frozen by immersion in liquid nitrogen. The samples were then placed in a Bright 5030 cryostat at -23 °C. Sections with 10 µm thickness were collected on glass slides, and stored at room temperature until examined. Staining with 0.1% TB was used directly on the sections with the section being covered in stain, then covered with a coverslip, and lastly washed in water under the coverslip before examination. Sections were imaged with a Leica DFC 450C digital camera equipped with x5 and x10 objectives. Images taken represent the muscle structure and the overall appearance of the structure, including spaces, adipose tissue, connective tissue, hyaline cartilage and bone particles. In addition, the percent of intact muscle was estimated (Table 2).

The Danish method

The samples were thawed, and fixed in formalin, embedded in paraffin, and 3 µm thick sections were cut prior to staining. For myosin, an antibody titer of 1:100 diluted in Dako antibody diluent S0809 with heat-induced epitope retrieval (HIER)

Table 2. Grades of MSM according to the UK method, and correspondence to % intact muscle.

Scale	% intact muscle
1	100-75
2	75-50
3	50-25
4	25-0

in Cell Conditioning 1 at pH 8.5 at 99 °C for 48 min, and for laminin, an antibody titer of 1:2 diluted in Dako antibody diluent S0809 with proteolytic pretreatment with protease 1 (Ventana) at 36 °C for 24 min was used. OptiView-DAB 750-700, BenchMark Ultra Ventana was used as detection system. Histological assessment was conducted on a fully automated NIKON COOLSCOPE microscope sufficed with a digitalized stage board, a camera, and Checkvision software able to measure the content of myosin and the relative content of structured muscle tissue relative to the sample size and to the muscle content. Magnification of x5 was used, and 15 randomized images per sample were acquired.

Table 3. Linearity between the relative amount of meat and the measured amount of intact fiber in the 11 mixtures with 5 replicates per mixture by the four histological methods.

Sample	German method	UK method	Danish method	HE
Thigh	0.61	0.85	0.94	0.62
Breast	0.73	0.98	0.91	0.73

III. RESULTS AND DISCUSSION

The assessment of reference samples from breast and thigh meat showed an overall decreasing trend in the grade regarding content of structured muscle fiber with decreasing amounts of minced and increasing amounts of emulsified meat in the German, Danish, and the alternative method. In the UK method, a similar trend was seen in the thigh samples, but not in the breast, although it was expected because breast muscle should be more prone to degradation due to the muscle type [7]. However, the German and HE staining showed a rather large variability between sub-samples, while the UK method had a very good correlation in the breast muscle but larger variation in the thigh muscle. Laminin showed acceptable level of accuracy (data not shown).

Table 4. Correlation between the histological methods.

	Method	HE	German	UK
Thigh	Danish	0.87 (n=11)	0.89 (n=11)	0.78 (n=11)
	HE		0.92 (n=11)	0.73 (n=11)
	German			0.73 (n=11)
Breast	Danish	0.86 (n=11)	0.88 (n=11)	0.93 (n=11)
	HE		0.94 (n=11)	0.88 (n=11)
	German			0.83 (n=11)

Thus, these methods showed a variable capability to predict the amount of structured muscle in the sample (Table 3).
Correlation between the histological methods exposed that the German and HE method, and laminin correlated relatively well (r=0.86–0.89) for both breast and thigh, whereas the correlation with the UK method was good for the breast reference samples (r=0.83-0.93) but much lower for the thigh (r=0.73-078) (Table 4).

IV. CONCLUSION

The antibodies used in the Danish method could be used on chicken material to visualize myosin, and hereby estimate the content of muscle tissue in the sample, and to determine the laminin content in order to evaluate the level of degradation of the muscle tissue. The four histochemical methods showed different capabilities to predict the amount of structured muscle in the reference samples. The UK method showed a very clear prediction in the breast meat, whereas this was not the case for the thigh samples. The Danish method showed a little lower capability of prediction of structured muscle; however, it was able to do so both in the breast samples as well as in the thigh samples. The German method and HE showed too high variability in the replicates, thus the prediction capability was rather low.

The correlation between the methods was

relatively high for the breast reference samples between all the methods, but for the correlation between the thigh samples there was a lower correlation between the UK method and the other methods.

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