The optimisation of the bone fragment content determination in histological sections

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Abstract

This article describes the optimisation of the histological procedure for the analysis of bone tissue in meat products as evidence of mechanically separated meat (MSM). The results of the analysis of ten products show differences in the interpretation of results between the method of determining the number of bone fragments on ten sections and the method of determining bone fragments on a defined area. The analysis of serial sections with respect to the size of bone fragments also shows that there should be a distance of 0.6 mm between individual sections and that a group of bone fragments within a distance of 0.3 mm from the centre of the given group should be counted as a single bone fragment.

Bones, histochemistry, histology, mechanically separated meat, mechanically reclaimed meat, shattered bone

Introduction

A large number of methods are used to provide detection of MSM. At the present time, some of methods are either in development or are experimental. Methods are based on the detection of damage to muscle tissue, on chemical composition (calcium, phosphorus, cholesterol, fatty acids, protein composition) or water activity, while immunological, PCR, textural, rheological and histological methods are also used. The principal reason for the use of such a wide range of methods is the ambiguity of individual tests for the identification of MSM. A method based on the identification of calcium by atomic absorption spectrometry is currently used in the Czech Republic, and this should be confirmed by at least one other test (EFSA 2013). Surveillance authorities generally use histological methods based on the work of Tremlová and Štarha (2003) to confirm the presence of MSM. This method is based on the use of special staining alizarin red S Alizarin red S forms chelate complexes with calcium that are differentiated from other structures by contrast (a red colour). To ensure the accuracy and, first and foremost, comparability of the results of histological tests, it is essential that all inspection laboratories observe the principles of microscopic laboratory practice and use a uniform methodology for the interpretation of the results. Errors in the interpretation may be caused by the duplicate counting of bone fragments, i.e. a single bone fragment being counted more than once, or by splinters of a single bone fragment being counted as more than one bone fragment. Bone fragmentation occurs during the production of MSM. Various technologies are used for the production of MSM, each of which produces MSM of differing parameters. The decisive thing for our purposes is the size of the bone fragments. In belt-drum systems, MSM is squeezed into a stainless steel drum through 1 – 10 mm holes and subsequently through a sieve with 1 – 2 mm openings for final deboning (Field 2004). In screw separators, the raw material (poultry skeletons, wings, etc.) destined for separation is first cut up into small pieces which are then squeezed by a screw conveyer onto a separation filter with approximately 0.5 mm openings (Barbut 2002). Another type of separator used is a linear pressure separator, used for the separation of red meat, poultry and fish. The first step in the separation process is, similarly to that for

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the screw conveyor, the raw material being cut up into small pieces (10 – 15 mm) which are then squeezed in a piston-like device and pass through a separation filter with openings 1 – 1.3 mm in size (Field 2004). Another possible error in the interpretation is caused by the varying size of the sections examined which leads to differing results as to the number of bone fragments per section. The aim of this study is to point out possible errors in the interpretation of the results of histological tests of bone tissue and to propose a procedure that will lead to reproducible results and prove comparable with the results of the tests used in European food laboratories.

Materials and Methods

A total of 10 samples of meat products were tested, of which 7 contained MSM and 3 did not contain MSM (samples E, I and J). Samples A, B, E and F were of cooked sausage, C and D of frankfurters, and samples G, H, I and J of hamburgers. Four paraffin blocks were prepared from each sample, from which ten histological sections were prepared. The samples were processed using the paraffin embeading histological technique, section were cut on an RM 2255 Rotary Microtome (Leica, GER) to a section thickness of 4 μm with a distance between sections of 50 μm. Alizarin red S was used for staining. Procedures for histological staining are based on a traditional staining procedures modified for food matrices. The staining procedures are given in the "Manual of methodologies for foodstuff histology" (Manual 2005). The stained sections were examined in an Eclipse E220 light microscope (NIKON, JPN) at 40 and 100 times magnification.

In the 10-sections method of determining the number of bone fragments, the numbers of all bone fragments were added together and the result interpreted as positive (evidence of the presence of MSM) if more than three bone fragments were found. In the defined-section area method, the number of bone fragments was related to the surface of the section measured, and the result expressed as the number of bone fragments per 1 cm^2. Using this method, the presence of < 0.2 bone fragments is considered a negative result, 0.2 – 1.5 dubious and > 1.5 positive (Ketteritzsch 2007). A result for which we cannot declare with a high degree of certainty that MSM has been used in the product is considered a dubious result. Bone tissue was identified by red precipitate. Serial sectioning of samples was employed for the evaluation of duplicity and the splintering of bone fragments and the samples arranged according to contact points into a sequential series of images by the programme RegEdit (Janáček, CZE). Analysis itself was performed by the program Ellipse (ViDiTo, SVK); a 3D model was created by the module Surface (Janáček, CZE).

Results and Discussion

The results and discussion are divided into two parts for the sake of clarity. The first part compares the limits used to demonstrate conclusive evidence of bone fragments in the Czech Republic with an interpretation of the number of bone fragments in a defined area used abroad (Ketteritzsch 2007). The second part considers the duplicate counting of a single bone fragment more than once.

The interpretation of the number of bone fragments in a defined area

The limit used for confirmation of the suspected use of MSM in the Czech Republic is three bone fragments in 10 sections. It must be noted that this method does not consider the size of the area of the sections and is based merely on usual laboratory practice in the preparation of histological sections. Similar approaches to the interpretation of these results are also mentioned in the literature. Bijker et al. (1985) stipulated a limit for conclusive evidence of MSM of 60 bone fragments in eight sections, a value of 30 – 59 as a dubious result, and a figure beneath 30 as negative. With the development of new MSM production technology, however, this procedure has been validated by Schulte-Sutrum and Horn (2003), who proposed a lower limit for the confirmation of MSM use in a product. They stipulated a limit for confirmation of MSM use of 1.5 bone fragments, a dubious result as 1 – 1.5 bone fragments, and less than 1 bone fragment per section as negative, with the minimum number of sections examined stipulated as ten. The occurrence of one bone fragment per section should also be tolerated according to Stenzel and Hilderbrant (2006).

The interpretation based on the number of bone fragments is, however, problematic for the purposes of comparison with the results given in the literature or for inter-laboratory comparisons. The usual sample size for histological examination is 1 cm^3. We do, however, also come across samples of a size of 3 cm^3 resulting from the maximum size of tissue blocks of 2 x 3 x 0.5 cm. We can, therefore, obtain sections of a size of 1 cm^2 or of 6 cm^2. The total area may be even smaller in the case of crumbly samples, samples that prove hard to fix or samples of small size. It is appropriate, for these reasons, to interpret the results according to the size of the area actually analysed. Other studies and the methodical instructions of another surveillance authorities (Ketteritzsch 2007) use the number of bone fragments per 1 cm^2 for the purposes of evaluation.

Ten samples were tested for the purposes of comparison of two methods, of which seven contained MSM and three had no addition of MSM. The use of MSM was not confirmed in the samples with no addition of MSM (meat product E, hamburgers I and J) by either method. The results of two compared methods did not be in conformity with added MSM. All these samples were recorded as positive according to the interpretation used in the Czech Republic. The method employing the calculation of the number of bone fragments per 1 cm^2 evaluated 3 of the samples as positive and 4 samples as dubious. This finding shows that the interpretation from these methods are different (Table 1, Samples A, B, G and H). The reason for this difference is the fact that the analysed section area is not taken into consideration in the case of the method used in the Czech Republic (10-sections method). The low spread of the number of bone fragments in products B, D and E shows that the boundary limit for dubious results cannot be determined for the method evaluating the number of bone fragments on ten sections for the reason of the differing area analysed.

The determination of the boundary of dubiousness, however, increases the accuracy of the interpretation of the results. Where dubious results are obtained, this test must be supplemented by additional results obtained using another method, such as AAS for the determination of calcium. The reverse is, of course, also true when a histological method is used as a supplementary method as recommended by Branscheid et al. (2009) and Ketteritzsch (2007).

Table 1. Detection of MSM by the method with a limit of 3 bone fragments per 10 sections

Sample	Number of bone fragments/10 sections	Evidence of MSM according to the limit used
Product A	36	positive*
Product B	25	positive*
Product C	82	positive
Product D	27	positive
Product E	0	negative
Product F	28	positive
Hamburger G	9	positive*
Hamburger H	3	positive*
Hamburger I	0	negative
Hamburger J	1	negative

*samples with presence of bone fragments confirmed with merely differing result interpretation

Duplicate counting of bone fragments

Bone fragments in MSM may be of various sizes depending on the method of separation used. The size of bone fragments in the final product should not be more than 0.5 mm for

Table 2. Detection of MSM by the method with a defined section area

Number of bone fragments/1 cm^2	Evidence of MSM according to the recommended limit
1.21	dubious*
1.00	dubious*
14.85	positive
9.45	positive
0.00	negative
2.70	positive
1.32	dubious*
0.78	dubious*
0.00	negative
0.07	negative

*samples with presence of bone fragments confirmed with merely differing result interpretation

conveyor separators or 1.3 mm for screw separators. This statement agrees with the results of Koolmes et al. (1986), who analysed MSM from 16 separators and showed an average bone fragment size ranging from 0.1 to 1 mm in 91.3% of bone fragments. The valid Czech legislation and European regulations do not stipulate a maximum size for bone fragments in MSM. The US legislation takes a different view of this matter, permitting the occurrence of bone fragments to a maximum size of 0.85 mm, with 90% of bone fragments having to be of a size of no more than 0.5 mm. The study by Tremlová and Štarha (2003) confirms that the majority (79%) of bone fragments in MSM are up to 0.5 mm in size, 10% of bone fragments of between 0.5 and 1 mm in size, with only 0.2% of bone fragments being larger than 1 mm. These results were subsequently confirmed by the work of Čáslavková et al. (2012), who state that 99.5% of bone fragments analysed were less than 0.5 mm in size. Only 0.5% of bone fragments were larger than the given limit of 0.85 mm, with 0.1% of bone fragments larger than 1 mm.

The size of bone fragments also has an influence on the interpretation of the number of bone fragments in histological sections. If large bone fragments occur, they may be counted more than once (Plate IX, Fig. 1) in serial section. It is necessary, for this reason, to leave a distance between individual histological sections when preparing samples for analysis. In view of the size of the majority of bone fragments, a distance larger than 0.5 mm between sections can be recommended. In view of the thickness of tissue blocks of 20 mm, a distance of 0.6 mm can be recommended. In this case the probability that we will not come across a bone fragment larger than 0.5 mm, which is the value taken as significant according to the statistical calculations, is 99.5%.

Another reason for the duplicate measurement may be the counting of individual parts of a single bone fragment that may look like a cluster of various bone fragments due to the greater splintering of a piece of bone (Plate IX, Fig. 2). With a view to due caution, it can again be recommended that bone fragments at a distance of no more than 0.3 mm (Plate X, Fig. 3) from the centre of a given group of bone fragments be counted as a single bone fragment with the same probability. An example is shown in (Plate X, Fig. 4), in which a 3D model has been created on the basis of analysis of the radius in serial sections of a bone fragment of a size of 0.5 x 0.7 mm, documenting the interconnection of individual splinters of a single bone fragment.

Conclusions

The results of this study show that a standard evaluation procedure must be introduced with a view to inter-laboratory comparison of a histological method for demonstrating the

presence of bone fragments which can be used as an indicator of the use of MSM. The study proposes the interpretation of the number of bone fragments per 1 cm^2. The resulting number can be divided into individual categories on the basis of the results produced by the authors and foreign literary sources as follows in accordance with the number of bone fragments per 1 cm^2: negative (< 0.2), dubious (0.2 – 1.5) and positive (> 1.5). In view of the size of bone fragments, it is recommended that tissue blocks be prepared with a distance of 0.6 mm between individual sections and that groups of bone fragments at a distance within 0.3 mm of the centre of a given group of bone fragments be counted in all cases as a single bone fragment.

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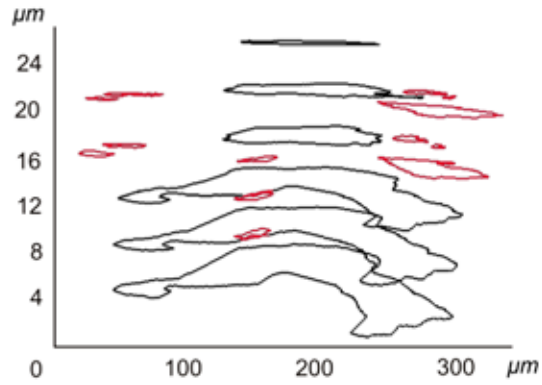

Fig 1. The arrangement of a bone fragment in a product with clear segmentation shown in red (serial section)

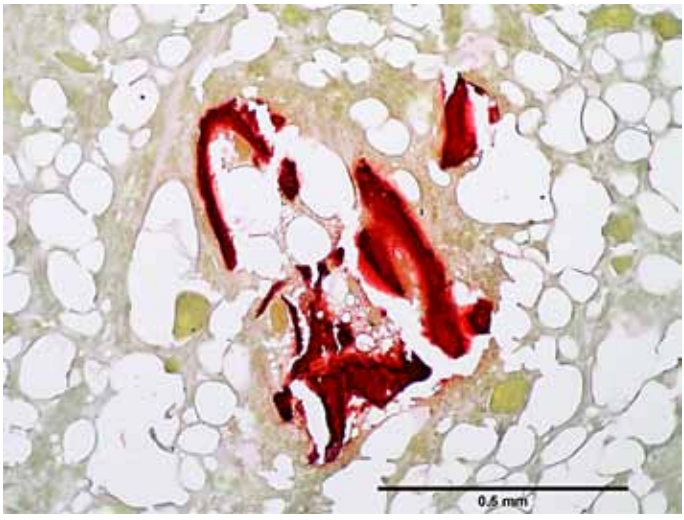

Fig. 2. The splintering of a bone fragment

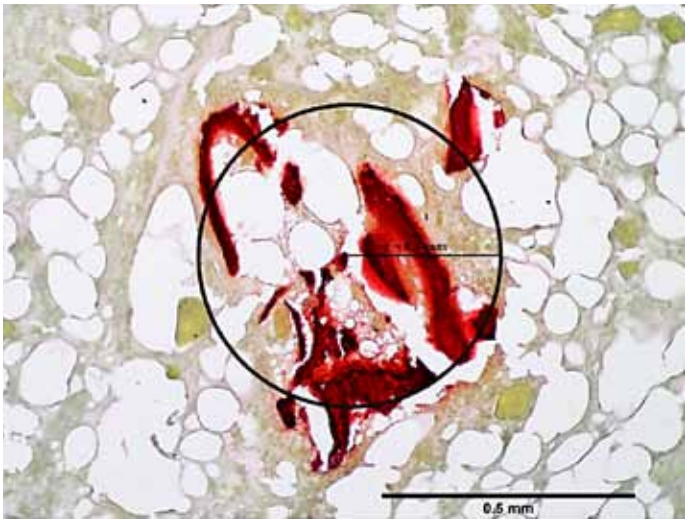

Fig. 3. Distance for counting parts of bone fragments as a single bone fragment (r = 0.3 mm)

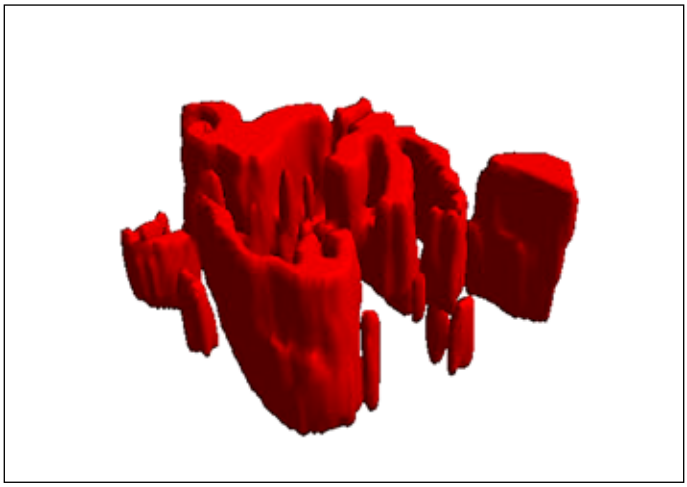

Fig. 4. A 3D view of the splintering of a bone fragment