Antigen retrieval and fixation of sections on slides for immunohistochemical detection of soya protein in meat products

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Summary

Soybean protein is added to meat products as a cheaper substitute of muscle proteins. If the presence of soybean protein in meat products is not stated on the label, this can be taken as adulteration. Detection of soybean proteins in foodstuffs is important in case of a higher rate of their application. This work was focused on the influence of solutions acting as unmasking buffers for soybean antigen retrieval in immunohistochemical examination of meat products. Seven buffers (solutions) with different pH, chemical composition or molarity were tested. The citrate EDTA (ethylenediaminetetraacetic acid) buffer facilitated the highest efficiency in soybean antigen retrieval, while 5% (w/w) urea + H_2O the lowest one. Simultaneously, the effect of these buffers on section fixation on slides was tested. The highest efficiency for fixation of sections on slides was recorded for 4% (w/w) $AlCl_3$ + Tris-HCl (tris(hydroxymethyl)aminomethane-HCl) ($p < 0.05$), which reached the lowest efficiency in terms of antigen retrieval. The efficiency of citrate buffer in antigen retrieval (immunolabelling intensity) was strong and fixation of tissue sections on slides was high ($p < 0.05$). Therefore, citrate buffer was found to be optimal and can be recommended for immunohistochemistry of meat products.

Keywords

unmasking; buffer; epitope; immunolabelling

Soybean protein is currently a common ingredient of some meat products. Soya is added to meat products in the form of isolates, concentrates or protein, in particular for technological reasons (it increases the binding capacity for water) but also for economical reasons (it is a cheaper substitute for muscle proteins). Under the Regulation (EU) No. 1169/2011 of the European Parliament and of the Council [1], soybeans and food products of them have to be labelled. The necessity of soybean protein detection follows from the high rate of application of this protein in meat products. Methods of soybean protein detection include enzyme-linked immunosorbent assay (ELISA), molecular genetics methods, chemical, physical and imunohistochemical (IHC) methods that, as described by POSPIECH et al. [2], are highly sensitive and can detect the presence of soya protein at a level of 0.1% (w/w) [2–5].

The problems in the detection of protein additives of plant origin in meat products follow from their application at low contents, but also from modification of their structure by the manufacturing process [2, 6]. Maintaining the tissue structure and antigenic reactivity is necessary for IHC binding. These two requirements are often in conflict with each other because the effective fixatives for tissue structures often change biological activity of the protein, such as enzyme activity and antigenicity [7]. Reverting structural changes in molecules to the original state can be performed applying the techniques of antigen retrieval, antigen unmasking or antigen revitalization, as it is called in some sources as well, which improves the detection of antigen epitopes by increasing the visibility of details on the tissue surface and precise localization of the antigen in the examined tissue [8].

In recent years, several methods supporting the

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accuracy of IHC detection were used for antigen retrieval [9–14]. Nowadays, antigen retrieval procedures are based on two mechanisms of action, enzymatic method using proteolytic enzymes and a non-enzymatic method. The most progressive non-enzymatic technique of antigen retrieval from tissues fixed in formaldehyde is the heat-induced epitope retrieval (HIER) [12, 13]. In general, the mechanism of heating effect influencing the antigen retrieval is unclear. Nevertheless, the result is a reversion of structural changes incurred during fixation, extraction of diffusion blocking proteins, protein precipitation and rehydration of a part of the tissue occur, which allows for better penetration of antibodies into the tissues [15].

To achieve the optimal results in the IHC laboratory, every newly introduced method (in our case, the detection of soybean antigen) in meat products should undergo a testing process to find the best system for the specific antibody, fixation and the type of processing, in particular the type of buffers for incubation and their chemical composition, pH and molarity [7, 12].

Another problem of IHC examination addressed by several studies is the fact that damage or loss of the sections from slides and also extensive modifications in the structure of proteins occur, which often adversely affects the antigenic properties of the protein and the sought antigens are then difficult to be retrieved, which may in some cases lead to inaccurate and distorted results of the immunohistochemical examination [15].

The aim of this study was to evaluate the influence of seven buffers on the antigen retrieval of soybean protein associated with immunolabelling intensity as well as to determine the effect of unmasking buffers on fixation of meat product samples on slides.

MATERIAL AND METHODS

Preparation of samples

A dry sausage (Technological workroom for meat processing, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic) with 1.5% soybean protein additive was manufactured based on commonly available recipes [16].

Sample treatment and preparation

For sample preparation, procedure according to Pospiech was applied [2]. Three samples in a size of 1 cm^3 were collected and placed in a fixative (10% formaldehyde) for 24 h, then purified in water (30 min), drained in autotechnicon (Leica TP 1020, Wetzlar, Germany) and embedded into paraffin blocks in Paraplaste (RNDr. Jan Kulich, Prague, Czech Republic). These were subsequently cut to 4 μm sections in a rotation microtome (Microm HM 400; Carl Zeiss, Jena, Germany). Three sections were mounted to each slide (SuperFrost plus; Menzel-Glaser, Braunschweig, Germany).

Examination procedure

In each examination, which was repeated ten times with a frequency of once a week, nine sections from each unmasking buffer (63 sections per day), were evaluated. The total number of 630 sections were immunohistochemically processed and subsequently microscopically examined at × 40, × 100 and × 400 magnifications in a light microscope Nikon ECLIPSE E200 (Nikon-Alphaphot-2 YS 2, Nikon Type 119; Nikon, Tokyo, Japan). For documenting the results, a set of digital photographs was captured by means of Canon Powershot A620 camera (Canon, Tokyo, Japan) utilizing image capturing software PSRemote Version 1.5.2 (Breeze systems, Bagshot, United Kingdom). Individual sections were also scanned using Super CoolScan 9000 ED (Nikon) with a microslide adapter, and subsequently processed in the image analysis software ACC (Adaptive Contrast Control Structure and Object Analyser version 6.1; ACC Sofo, Brno, Czech Republic), the whole section being set as the total section area (100%). The fixed area percentage after processing was marked and measured using tools of ACC. The portion of the section area applicable for the analysis was also assessed.

To interpret the results, qualitative analysis by graphical representation was used. Poor immunological response (weak immunolabelling intensity of the soybean antigen) was marked as (+), distinct as (++), and strong as (+++). This way is commonly applied for evaluation of immunohistochemical methods for example by Okada et al. [14] and Emoto et al. [17].

Immunohistochemical examination of samples

For immunohistochemical detection, a three-stage indirect method using streptavidin-biotin-complex (ABC) was applied. In this method, the primary antibody binds the secondary antibody conjugated with biotin. In the next step, the secondary antibody binds the streptavidin-biotin-peroxidase complex, which greatly amplifies the immunolabelling signal. In this research, a primary polyclonal antibody against soybean protein Anti-Soy Protein S2519-1 (Sigma-Aldrich, St. Louis, Missouri, USA) was used. It is a poly-

Tab. 1. Comparison of buffers at antigen retrieval.

No.	Buffer	pH	Replicate									
			1	2	3	4	5	6	7	8	9	10
B1	4% (w/w) $AlCl_3$ + Tris-HCl	3.5	+	+++	+++	+	+	+	+	++	+	++
B2	4% (w/w) $AlCl_3$ + H_2O	3.2	+	++	++	++	+	+	+	+++	+	++
B3	1% (w/w) $ZnSO_4$ + H_2O	5.7	+	++	+	+	+	++	+++	++	++	+
B4	5% (w/w) urea + H_2O	7.0	+	++	++	+	++	+	++	++	+	+
B5	Citrate buffer	6.0	++	++	+++	+++	+	++	+	++	++	++
B6	2 mol·l^{-1} Gu-HCl + Tris-HCl	9.1	+	++	+++	++	+++	++	+	+	+++	++
B7	Citrate-EDTA	6.2	++	+++	++	+++	++	+++	++	+++	+++	++

Immunolabelling intensity of soybean antigen: (+) – weak; (++) – distinct; (+++) – strong.
Tris – tris(hydroxymethyl) aminomethane), Gu – guanidine, EDTA – ethylenediaminetetraacetic acid.

clonal fractionated antiserum in a buffered aqueous solution obtained by immunization of rabbits. A dilution of 1:500 for primary antibody was utilized according to previously published methodology [2]. Biotinylated antibody (PK 6101; Vector Laboratories, Burlingtone, Massachusetts, USA) was used as the secondary antibody. In the third stage, ABC reagent was used.

For antigen retrieval, a technique using microwave tissue heating for 5 min at 650 W in one of seven unmasking buffers was applied. The seven unmasking buffers differed in pH, chemical composition and molarity (Tab. 1). Each examination was carried out repeatedly, specifically ten times on ten different days. Within the analysis, nine sections for each unmasking buffer were processed.

Preparation of individual buffers was performed in the laboratory in accordance with the stated procedures. After preparation, buffers were filtered and pH was adjusted to the desired value. All buffers, except for 2 mol·l^{-1} GuHCl (guanidin-HCl) + Tris-HCl (tris(hydroxymethyl)aminomethane-HCl), were stored in a refrigerator all over the time. Prior to each analysis, pH was adjusted to the desired value.

Statistical analysis

The results were processed using Minitab software (State College, Philadelphia, Pennsylvania, USA) by means of Pearson's test of independence and Kruskal-Wallis test of equality of medians.

RESULTS AND DISCUSSION

Antigen retrieval

The effect of unmasking buffers on antigen retrieval and immunolabelling intensity of soybean antigen was tested. The immunolabelling intensity of IHC reaction for the applied buffers is shown in Tab. 1. In accordance with Shi et al. [18], our results indicated a significant effect of pH of the unmasking buffers. Effect of pH value of the incubation solution for the antigen retrieval and, subsequently, on the immunolabeling intensity, was described in several studies [14, 17, 19, 20]. In agreement with these studies, pH value had a stronger impact on antigen retrieval than the chemical composition of the buffer (Tab. 1). These results also show that buffers with pH from 6 to 9.1 facilitated better immunolabelling intensity of soybean antigen in meat products. This was due to unmasking buffers, which retrieved antigen epitopes and, consequently, the antigen-antibody bound more easily to the appropriate soybean antigen in dry sausage. This concerned the following buffers: citrate buffer, pH 6 (B5); Gu-HCl + Tris-HCl, pH 9.1 (B6); and citrate-EDTA (ethylenediaminetetraacetic acid), pH 6.2 (B7).

Buffers with pH from 3.2 to 5.7 ($AlCl_3$ + Tris-HCl, pH 3.5; $AlCl_3$ + H_2O, pH 3.2; $ZnSO_4$ + H_2O, pH 5.7) facilitated only low immunolabelling intensity of soybean antigen. Detection of the antigen-antibody bond in samples incubated in these buffers was weak. It can be concluded that the antigen retrieval by these buffers was inferior to buffers with a higher pH value (6–9.1). This can be explained by the fact that the isoelectric point of polyclonal antibodies is in the range of pH 6–9.5 and, within this pH range, the immunological reaction antigen-antibody is more intense [15].

The results on pH dependence of the antigen retrieval support the assumption that heating cleaves intermolecular and intramolecular cross-links in proteins and extends polypeptide chains. Polypeptides are charged negatively or positively in the alkaline or acidic pH, and electrostatic repulsion prevents accidental entanglement of polypeptides under the influence of intermo-

lecular forces, and, in the section cooling process in incubation buffers, the sought antigens exhibit their epitopes. In contrast, at pH from 4.5 to 7.5, electrostatic and hydrophobic attractions act in co-operation with the adjacent polypeptides, the molecules are entangled with each other and thus their antigen epitopes are hidden [11].

Furthermore, similar results were obtained with citrate buffer (pH 6) and with Gu-HCl + Tris-HCl with pH 9.1 (B6). Citrate buffer is the one most commonly used for antigen retrieval and, in a number of studies, it is used as the reference buffer, being assumed to effectively break cross-links between calcium ions and protein [12–14, 19, 21, 22]. Ramos-Vara [15] reported that citrate buffer provided satisfactory results and also allowed for a clear resolution of cell morphology compared to buffer systems with pH 6 and higher. Nevertheless, this was not confirmed in our research. The reasons may include the fact that in food microscopy, lower resolution and magnification (100- to 400-times) is sufficient for examination of foodstuffs, unlike in biopsy or studies at the level of cellular structures.

The best results, in terms of immunolabelling intensity and the associated best ability to unmask antigen epitopes in meat products, were obtained with the citrate EDTA buffer (B7, pH 6.2; Tab. 1). Amplification of the antigen-antibody bond was strong and this buffer was the most often evaluated with three graphic signs (+++) as to the intensity of immunolabelling. Citrate EDTA has a similar pH value as citrate buffer, but it seems that its different chemical composition has a decisive impact on its unmasking ability of antigen epitopes in dry sausage. In the detection, citrate-EDTA facilitated the strongest immunolabelling intensity, the background being not stained at all, which is consistent with Eisen [12]. Tissue-bound calcium ions might be important in masking some antigens during fixation [15]. Calcium-chelating substances, including EDTA, have the ability to bind calcium ions and thus unmask antigens [13, 15]. As shown in Tab. 1, the worst results of immunolabelling intensity were acquired by urea + H_2O buffer (B4). The immunological reaction between the soybean antigen and antibody was weak and almost unidentifiable. It should be noted that pH of this buffer was neutral (pH 7) and thus it was expected to show much better performance of immunolabelling intensity. Our results are in accordance with Shi et al. [21] who rejected the use of urea + H_2O as a standard buffer for immunohistochemical examination. This buffer seems therefore to be unsuitable also for IHC examination of meat products. According to Yamashita [11], buffers that are within the range of pH 4.5 to 7.5 have electrostatic and hydrophobic attractive forces. These are important for co-operation of adjacent polypeptides, which are mutually intertwined and, in this manner, they cover the antigen epitopes [11]. It should be noted that urea may, under certain circumstances, facilitate better results, for example in combination with 0.1 mol·l^{-1} Tris-HCl, pH 9.2 [21], but this was not verified in the present study.

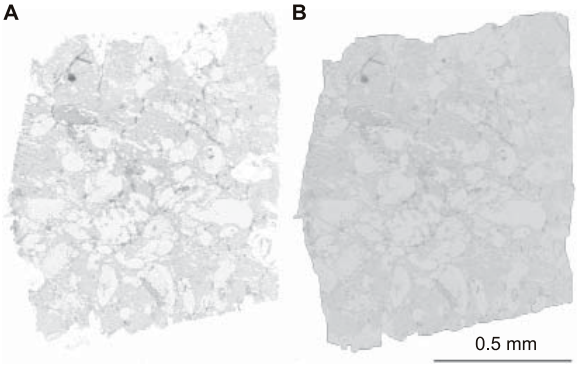

Fig. 1. 100% area fixation of the section on the slides.
A – unimpaired section, B – selected part of section.

The following solutions containing metal salts were tested next: $AlCl_3$ + Tris-HCl, $AlCl_3$ + H_2O, and $ZnSO_4$ + H_2O (B1, B2, B3). The reason was to verify the hypothesis of their favorable effects on IHC reaction, which was reported previously [9]. However, recently their use is being discontinued because it was found that metal salts are not an essential part of the antigen retrieval solutions used to unmask antigens, and, moreover, toxic effects of metal salts, in particular of lead salts, were discovered. All three tested buffers were characterized by acidic pH (3.5, 3.2, 5.7) and we confirmed that a lower pH is not optimal for immunohistochemical methods even in meat products. An acidic pH may cause weak false-positive reaction that may give rise to non-specific bonds in certain contact areas of the tested antibodies [19].

Fixation of sections on slides

In the following research, the influence of incubation buffers on fixation and stabilization of the sections on slides for immunohistochemical examination of meat products was studied. Based on the results obtained using the Pearson's test of independence and Kruskal-Wallis test of equality of medians, it was tested whether there were statistically significant differences between buffers in terms of section fixation on slides, i.e. if the

Tab. 2. Effectiveness of buffers in relation to 100% fixation.

Buffer	Number of examined sections	Number of 100% fixed sections	100% fixed sections [%]	95% confidence interval for proportions of 100%
B1	90	31	34.44	(24.74; 45.20)
B2	90	23	25.56	(16.94; 35.84)
B3	90	19	21.11	(13.21; 30.99)
B4	90	14	15.56	(8.77; 24.72)
B5	90	17	18.89	(11.41; 28.51)
B6	90	3	3.33	(0.69; 9.43)
B7	90	3	3.33	(0.69; 9.43)

Differences between buffers can be considered statistically significant if the interval estimates (range of two values) do not overlap each other.

applied buffer had any influence on fixation of sections on slides at all. Firstly, effectiveness of buffers in relation to 100% fixation (Fig. 1), i.e. the sections that remained fixed to slides fully was tested utilizing the Pearson's test of independence and, subsequently, the same was performed for effectiveness of buffers in relation to 0% fixation, in this case for buffers that caused total loss of sections from slides by washing off during incubation in buffers. In the first phase of the Pearson's test of independence, a hypothesis was tested that the proportion of 100% fixation in all 7 buffers was the same and, as we aimed to find statistically significant differences between the buffers, interval estimates with 95% confidence were calculated. As statistically significant were taken the results for buffers whose interval estimates did not overlap (range of two values in brackets in Tab. 2).

Tab. 2 shows that the buffer $AlCl_3$ + Tris-HCl (B1) was the most efficient out of all 7 buffers in relation to 100% fixation of sections on slides. The entire section area remained fixed in 31 cases (out of 90 tested sections). Buffer $AlCl_3$ + Tris-HCl was statistically different from buffers urea + H_2O, Gu-HCl + Tris-HCl, citrate-EDTA (B4, B6, B7) at a significance level of $p < 0.05$. In buffers B4, B6 and B7, 100% fixation of sections on slides was observed in 14 sections (urea + H_2O, B4) and in 3 sections for Gu-HCl + Tris-HCl, and citrate-EDTA. Using these buffers (urea + H_2O, Gu-HCl + Tris-HCl and citrate-EDTA) as fixatives proved to be unacceptable due to the greatest loss of sections from slides. Results for buffer urea + H_2O (B4) are consistent with the work of LUKÁŠ et al. [23] where 5% urea peformed the worst. The reason for this high loss of sections is the negative impact of urea on formalin-fixed tissues, because concentrated urea causes washing off formaldehyde and thus a low degree of fixation of sections on slides [23]. Buffers $AlCl_3$ + H_2O, $ZnSO_4$ + H_2O, and citrate buffer (B2, B3, B5) showed statistically significant differences ($p < 0.05$) compared with buffers Gu-HCl + Tris-HCl, citrate-EDTA (B6 and B7) and they were less effective than $AlCl_3$ + Tris-HCl (B1) by 13%.

We also used Pearson's test to find out whether there were statistically significant differences

Tab. 3. Effectiveness of buffers in relation to 0 % fixation.

Buffer	Number of examined sections	Number of 0% fixed sections	0% fixed sections [%]	95% confidence interval for proportions of 0%
B1	90	11	12.22	(24.74; 45.20)
B2	90	15	16.67	(16.94; 35.84)
B3	90	7	7.78	(13.21; 30.99)
B4	90	19	21.11	(8.77; 24.72)
B5	90	12	13.33	(11.41; 28.51)
B6	90	2	2.22	(0.69; 9.43)
B7	90	12	13.33	(0.69; 9.43)

Differences between buffers can be considered statistically significant if the interval estimates (range of two values) do not overlap each other.

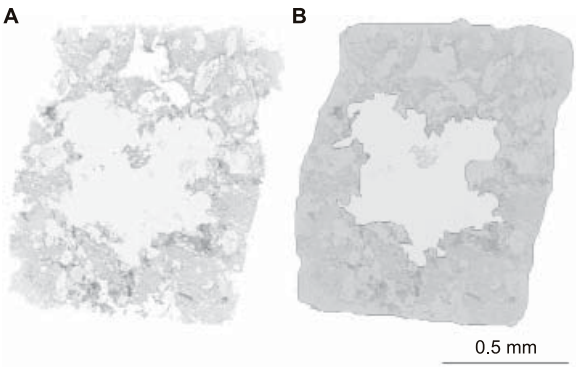

Fig. 2. 79% area fixation of the section on the slides.

A – impaired section, B – selected part of section (gray), impaired part (white).

between buffers in relation to 0% fixation, i.e. which buffers caused a total loss of sections from slides (Tab. 3). The results of this test again confirmed that buffers affected the fixation of sections ($p < 0.05$). In this case, the minimum loss of whole sections was reached by buffer Gu-HCl + Tris-HCl (B6), for which the total loss of 2 sections (out of 90 examined) was recorded. It differed statistically significantly from buffer urea + H_2O (B4), for which the loss of sections was detected in 19 cases. No statistically significant difference was demonstrated among other buffers ($AlCl_3$ + Tris-HCl, $AlCl_3 + H_2O$, $ZnSO_4 + H_2O$, citrate buffer, citrate-EDTA ($p > 0.05$). It can be concluded that, in these buffers, there was no difference in terms of the complete loss of sections.

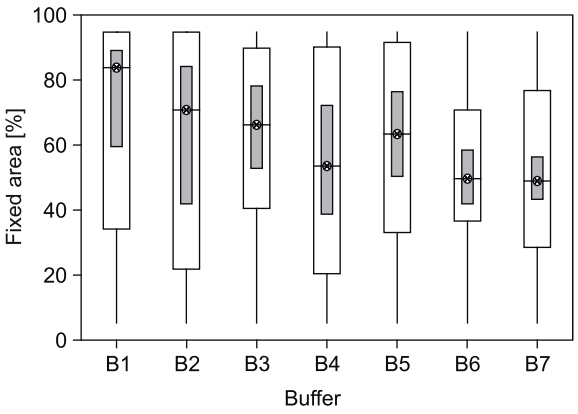

Fig. 3. Boxplot for median of percentages of fixed area sections on the slides.

Hatched rectangles (boxplot) are 95% confidence interval for median of percentages of the fixed area sections on the slides.

For comparison and assessment of the impact of buffers not only with respect to 100% and 0% fixation of sections on slides, the test of equality of medians (Kruskal-Wallis test) was used. After IHC examination only a part of some sections remained, i.e. sections were damaged or torn (Fig. 2). The researcher should, however, be aware of whether to include the damaged sections in the overall evaluation and interpretation of results. In this work, the damaged sections were also included in the overall results. The graphical representation of the results of percentages of fixed area sections on slides was performed as a boxplot or box diagram, which is a common approach in descriptive statistics. In Fig. 3, the central "box" part of the diagram (50% using values) is bounded by the third quartile (on top), first quartile (down) and between them is the line delimiting the median. The results of the test of equality of medians clearly showed that, based on the entire area of sections fixed on slides, the most effective buffer was $AlCl_3$ + Tris-HCl (B1), which was statistically different from buffers Gu-HCl + Tris-HCl and citrate-EDTA (B6, B7) at a significance level of $p < 0.05$ (Fig. 3). Differences in the effectiveness of buffers $AlCl_3 + H_2O$, $ZnSO_4 + H_2O$, urea + H_2O and citrate buffer (B2, B3, B4 and B5) were not statistically significant. Results on the equality of median confirmed the results of Pearson's test in relation to 100% fixation. In both tests, the most successful fixation solution proved to be buffer $AlCl_3$ + Tris-HCl (B1) that achieved the best performance not only in relation to 100% fixation (complete sections fixed), but also when the entire area of sections was taken into account (total), including damaged sections with only a certain part fixed. Likewise, the results of both tests were identical in stating that the worst fixation on slides was obtained with buffers Gu-HCl + Tris-HCl and citrate-EDTA (B6 and B7; $p < 0.05$).

The results of our study do not confirm the view that the pH value has a significant effect on fixation of sections on slides [24]. The best efficiency was demonstrated by buffer $AlCl_3$ + Tris-HCl with a higher, acidic pH (3.5), which was better than buffers with higher or alkaline pH values: citrate-EDTA (pH 6.2) or Gu-HCl + Tris-HCl (pH 9.5). It is necessary to mention that citrate-EDTA (pH 6.2) has a pH value quite similar to citrate buffer (pH 6). However, citrate EDTA had statistically significantly the lowest efficiency in fixing sections on slides, unlike citrate buffer with average results that did not differ significantly from other buffers tested and was acceptable for IHC detection. Based on our results, the hypothesis of an optimal pH range, which would have a major

impact on sections fixation on slides for immunohistochemical examination of meat products, cannot be confirmed.

Loss of sections from slides and changes in the structure of molecules arise mainly during fixation and further processing [25]. After fixation (in formaldehyde or glutaraldehyde), the paraffin-embedded tissue sections commonly used in histological studies are dehydrated in alcohol, purified by xylene and embedded in paraffin at 55–60 °C. Ramos-Vara [15] stated that the loss of sections from slides may be affected by unmasking buffers in which the sections are incubated. This fact was confirmed in meat products by Řezáčová-Lukášková et al. [6], who tested five different methods of slide coating, including poly-L-lysine and described another way to affect the degree of section damage during IHC.

CONCLUSION

In terms of section fixation on slides, the best results with statistical significance ($p < 0.05$) were reached by buffer 4% (w/w) $AlCl_3$ + Tris-HCl (B1) in contrast to buffers 5% (w/w) urea + H_2O, 2 mol·l^{-1} Gu-HCl + Tris-HCl and citrate-EDTA (B4, B6, B7). Results of 4% (w/w) $AlCl_3$ + H_2O, 1% (w/w) $ZnSO_4$ + H_2O and citrate buffer (B2, B3 and B5) were comparable. As for the influence of individual buffers on antigen retrieval (antigen unmasking), the best performance was observed at the buffer citrate EDTA, in which the highest immunolabelling intensity of soybean antigen-antibody binding was observed. Results of this research demonstrate that the impact of buffers on section fixation on slides and on antigen retrieval was incoherent. A suitable fixation solution for fixation of sections of dry sausage on slides was $AlCl_3$ + Tris-HCl (B1), but the best influence on antigen retrieval was shown by citrate-EDTA (B7), which, in terms of fixation, reached the highest losses of sections from slides. The results indicate that it is necessary to take both factors into account and, for these reasons, the citrate buffer is suitable for IHC of meat products. Citrate buffer guarantees acceptable (average) results in both aspects, i.e. regarding fixation of tissue sections on slides as well as antigen retrieval (immunolabelling intensity was sufficient). For this reason, it is advisable to recommend its application as a standard or reference buffer for immunohistochemical examination of meat products.

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