The use of special PAS Calleja staining for the microscopic detection of selected hydrocolloids in model meat products

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Abstract

Structure, composition and quality are extremely important in the production of meat products, for which reason many additives that have a positive effect on the properties of meat products are used in meat production. The aim of this work was to verify the suitability of special PAS Calleja staining for the detection of selected hydrocolloids in model meat products. Model meat products with a 1% addition of a selected hydrocolloid (carrageenans, potato starch, plant gums, xanthan and sodium alginate) were analysed. PAS Calleja staining was shown to be suitable for model meat products with added carrageenans and potato starch, with the given additive being stained an extremely intense purple-pink colour. A poorer colour effect was achieved in samples containing xanthan gum, gum tragacanth and sodium alginate, probably caused by the uronic acid content which results in aldehydes, which would react with Schiff's reagent, not being formed during staining.

Carrageenans, gums, histology, sodium alginate, starches

Introduction

The basic raw material in the production of meat products are various kinds of meat from slaughter animals. Blood and edible offal, obtained during slaughter processing and meat cutting, are secondary meat materials (Ingr 2011). Other ingredients and additives are added to meat products along with these primary and secondary materials. These additives may extend the shelf life of meat products, contribute to their structure and improve water-holding capacity (Kadlec et al. 2012). The structure, composition and quality of meat products have changed markedly in recent years. The principal reasons for this are the great competition among producers and the increasing pressure on lower product prices from the retail chains. Individual producers also differ in terms of the technological facilities at their disposal and the quality and origin of the raw materials they use. All the given parameters are then dependent on the technological process involved in the preparation of the batter of comminuted meat products (Budig and Mathauser 2007). A food additive is, according to Regulation (EC) No. 1333/2008 is a substance which is not usually intended for consumption as a foodstuff and is also not usually used as a characteristic constituent of a foodstuff, regardless of whether it is of nutritional value or not, and whose deliberate addition to food for technological reasons during production, processing, preparation, finishing, packing, transport or storage has, or will probably have, the consequence that this substance or its secondary products will become a direct or indirect constituent of this food.

Hydrocolloids are important food additives that profoundly modify the structural properties of foods as thickeners, stabilisers and gelling agents. Two types of hydrocolloid are found in foods – hydrocolloids usually present in foods (in particular, starch, gluten, cellulose, proteins and collagen) and additive hydrocolloids used to achieve the essential texture and functional properties of the food that are added deliberately to various foods

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to ensure the necessary structure of the final product (in particular, pectin, plant gums, carrageenans, modified starches, etc.) (Kalina and Váňa 2010). They are classed as polysaccharides, proteins or synthetic polymers in relation to their structure. Starch, cellulose and plant gums are examples of specific polysaccharides (Stephen 1995).

Starches are hydrocolloids which are used extremely often in the food industry. The principal reason for their use is their great ability to bind water (Luallen 2004). Because of this property, potato starch is used, for example, in the production of comminuted meat products (Murphy 2000). Starch can be combined with other hydrocolloids and proteins to obtain the desired yields and to influence texture and taste properties during the production of meat products (Pospiech et al. 2016).

Carrageenans are obtained by extraction with hot water at 74 °C in an alkaline environment in the form of sodium salts (extraction with solutions of sodium carbonate and sodium hydroxide). Acidic carrageenans are obtained by acidification with, e.g., hydrochloric acid. Gels are dried or precipitated with solvents after extraction (Kalina and Váňa 2010). The carrageenans used most widely commercially in the food industry are κ-carrageenan, ι- carrageenan and λ-carrageenan which are classed according to the degree of sulphation depending on whether they have one, two or three of the sulphate groups that influence their ability to form a gel (Cardoso et al. 2016). Carrageenans are used in meat products thanks to their thickening, gel-forming and stabilising properties. Their synergy with other hydrocolloids is another property of which advantage is taken. Unfortunately, this property cannot be used in meat products due to the high content of ions and the presence of animal proteins (Tarte 2009). The κ-carrageenan (gel-forming) usually dominates over λ-carrageenan (not gel-forming) at a ratio of 3:2 in commercial blends of carrageenans (Velíšek and Hajšlová 2009).

Guar gum is a polysaccharide obtained from the seeds of the plant *Cyamopsis tetragonoloba*. It is a galactomannan with a ratio of mannose to galactose of 2:1. It is used as a thickener and stabiliser. In meat products, it creates a smooth creamy feeling in the mouth similar to fats, for which reason it is often added to products with a low fat content in particular (Rather et al. 2016). Guar gum is well soluble in water and can be combined with the majority of natural gums, starches, pectin and cellulose. It is often used with xanthan, thereby increasing viscosity by dispersion (Velíšek and Hajšlová 2009).

Xanthan gum is a polysaccharide that is produced by the bacteria *Xanthomonas campestris*. It is used as a thickener and emulsion stabiliser, and forms a gel when mixed with other hydrocolloids (Velíšek and Hajšlová 2009). It is soluble in hot and cold water, even at low concentrations. Viscosity is increased by addition of the salt and the resulting solution is extremely stable even in the case of a change in pH or temperature. Xanthan gum can be used in products with a low fat content as an appropriate fat substitute (Rather et al. 2015).

Gum tragacanth is obtained from the branches of plants of the genus *Astragalus*. Its chemical composition, physical and physicochemical properties, and stability against temperature and low pH may differ depending on the individual species of plant. It contains two fractions: bassorin which swells in water and water-soluble tragacanthin (Karimi and Mohammadifar 2014). Gum tragacanth is stable against hydrolysis and mechanical stress. It is used as a thickener, emulsifier and stabiliser (Velíšek and Hajšlová 2009).

Alginates are extracted from brown seaweeds. Their structure is comprised of β-D-mannuronic acid and α-L-guluronic acid. Alginates form a transparent thermostable gel. Sodium alginate is the alginate used most widely in the food industry (Comaposada et al. 2015). Sodium alginate prevents the oxidation of fat during food storage, extends the durability of foods and preserves water and taste in foodstuffs (Yu et al. 2008).

The aim of this work was to find histological proof of selected hydrocolloids in model meat products using special PAS Calleja staining.

Materials and Methods

Model meat products with a 1% addition of selected additives (carrageenan, potato starch, xanthan gum, sodium alginate and plant gums – gum tragacanth and guar gum) were tested. Preparation of the model meat products began with the mincing of chicken breast muscle in a Thermomix 31 mixer (Vorwerk, Wuppertal, Germany) so that the muscle tissue was evenly comminuted. 1.5 g of kitchen salt, 0.5 g of polyphosphate and 10 ml of distilled water were then added to a weighed amount of chicken muscle. Kern scales (Kern & Sohn, Germany) were used for weighing, and all the ingredients were dry-mixed and transferred to a Vorwerk Thermomix 31 mixer (Vorwerk, Wuppertal, Germany) which homogenised the transferred mix thoroughly on setting 8 for a period of 2 minutes with the gradual addition of a measured amount of distilled water. The homogenised mix was transferred to a sample container of a volume of 250 ml. The mixture was pressed firmly to the walls of the sample container and covered with a polythene bag. The sample container with the prepared sample was placed in a holder in the middle of a ham cooker filled with water to the prescribed volume. The ham cooker was sealed with a spring lid. A thermometer was inserted into the centre of the ham cooker lid to allow the temperature at the core of the product to be monitored. The ham cooker with the prepared mix was placed in a thermostatic pot preheated to 90 °C. The temperature on the thermostat was reduced to 70 °C after a temperature of 70 °C had been reached at the core of the product, and the model meat product was then cooked for another 10 minutes. A combination of special PAS Calleja staining and light microscopy was chosen as the detection method. Four samples of a size of 1 cm^3 were taken from each model meat product and fixed in 10% formaldehyde and subsequently dewatered in a Leica TP 1020 tissue processor (Leica, Germany). Samples were sliced on a Leica RM 2255 rotary microtome (Leica, Germany) into slices 5 µm thick. Two slices selected at random were transferred to a single microscope slide after being floated out on water. Eight slices of each meat product were prepared for testing in this way. The slices prepared were placed for 2 hours on a heating plate set to 40 °C and then placed in a thermostat for a week. The next step was dewaxing in xylene and alcohol/ether. The slices were stained with special PAS Calleja staining. This was followed by dehydration and clarification. The procedure is described in Table 1 and the results of staining in Table 2. The next step was to mount the slices in Solakryl, after which they were studied under a Leica DM 3000 light microscope (Leica, Germany) and processed in the program XnView (Gougelet Pierre-Emmanuel, France). Eight slices of each meat product were studied in this way.

Table 1. PAS Calleja staining procedure

Procedure	Solution	Time
Dewaxing	Pure xylene	8 minutes
	Alcohol/ether (2/3 + 1/3)	8 minutes
Staining	Periodic acid	10 minutes
	Reducing bath	Rinse
	70% alcohol	Rinse
	Schiff's reagent	15 minutes
	Washing in running water	15 minutes
	Nuclei red	15 minutes
	Tap water	5 minutes
	Calleja B solution	5 minutes
	Distilled water	Rinse
Dehydration	96% alcohol	Rinse
	100% alcohol	Rinse
Clarification	Pure xylene	5 minutes
	Xylene for analysis	5 minutes
Mounting	Solakryl	

Results and Discussion

Special PAS Calleja staining, which employs the reaction between Schiff's reagent and aldehyde bonds in polysaccharides following hydrolysis with acids, was selected for the detection of individual food additives in model products (Rocha et al. 2012). The

Table 2. Staining results

Polysaccharides	Pink-red
Nuclei	Red
Muscle tissue	Green
Elastic connective tissue	Yellow
Collagen connective tissue	Blue
Plant protein	Dark green

formation of aldehyde bonds is depicted in (Plate IV, Fig. 1). Muscle tissue is stained green and polysaccharides a pink-red colour by this staining. There are at least eight sequences of carrageenans that differ in terms of the number and position of sulphate groups, though only three kinds of carrageenan – κ-carrageenan, ι-carrageenan and λ-carrageenan – are of importance in food (McClements 2005). Carrageenan Iota XE 4248 was detectable in all four blocks. The same was also true of the carrageenans Lambda XE 4405 and Kappa XE 4066 which were present in all slices. As is stated by Focke (2010), carrageenans form extremely firm thermostable gels that bind well to muscle tissue and make for the easy slicing of meat products. As can be seen in (Plate IV, Fig. 2), all the types of carrageenan used are bound to the skeletal muscle in the structure of the model meat product. The carrageenan bound water to itself which loosened its structure. Observed under the microscope, carrageenan can then be seen to occur in the meat product in places with air bubbles which are, in reality, areas formed where water has bonded. The basic chemical structure of carrageenans is formed by the disaccharide carabiose, which is formed of repeating sequences of β-D-galactopyranose and 3,6-anhydro-α-D-galactopyranose linked by a β-(1-4) bond. The individual carabioses are connected in a linear chain by an α-(1-3) bond (Velíšek and Hajšlová 2009). This disaccharide present in carrageenans was stained extremely well by the special PAS Calleja staining selected (Table 3) and this staining is suitable for the non-specific detection of carrageenans in model meat products.

Model meat products with a 1% addition of potato starch were then made in the second part of the study. PAS Calleja staining was also tried out on these model products. According to Černý (2007), starch increases the stability of meat products, forms gels and helps consolidate meat products by binding water during heat treatment. Another advantage it provides lies in improving the cohesion of the product while preserving its juiciness. The principal constituents of starch are two a-D-glucans – linear amylose with a-D(1→4) bound glucose units and branched amylopectin containing a-D(1→4) and a-D(1→6) bonds. The proportions of these polysaccharides, which differ in starches of differing origin, then influence a number of physical properties (Šárka et al. 2013). The amylose content in potatoes generally falls within a range of 17–21% (Pérez and Bertoft 2010). PAS Calleja staining, however, stains starches regardless of their amylose content, as is stated by Eliášová et al. (2012). As can be seen from (Plate V, Fig. 3), potato starch was stained a dark purple-pink colour by special PAS Calleja staining.

In the final part of this work, plant gums (gum tragacanth and guar gum), xanthan gum and sodium alginate were stained with PAS Calleja. The results of this staining are depicted in (Plate V, Fig. 4). All the plant gums tested and the sodium alginate were weakly stained. Xanthan gum has an irregular structure and is stained a blue-purple colour by PAS Calleja. It occurred at the edges of the model sample and in hollows. Guar gum was hardly stained at all by PAS Calleja. Gum tragacanth was, similarly to guar gum, also hardly stained at all. A meat product with the addition of sodium alginate at a concentration of 1% was also

tested. The alginate was stained a blue-purple colour. Similarly to xanthan gum, it has an irregular structure and occurred in the model product largely in hollows and at the edge of the slice. The probable cause of the weak staining of sodium alginate is that fact that it is formed of uronic acids. Uronic acids are sugar acids. They are formed of a monosaccharide whose hydroxyl group has oxidised at the primary end (Velíšek and Hajšlová 2009). The uronic acid content is also a possible explanation for the extremely weak staining of gum tragacanth and xanthan gum. In addition to arabinogalactans (tragacanthin), gum tragacanth also contains bassorin which is comprised of units of galacturonic acid which is also a uronic acid (Velíšek and Hajšlová 2009). The composition of sugar and the amount of uronic acids differs depending on the species of plants of the genus *Astragalus* from which gum tragacanth is made (Farzi et al. 2013). Xanthan gum is comprised of glucose and mannose, with glucuronic acid bound on a side chain (Rather et al. 2015). The principle of PAS Calleja staining is the oxidation of polysaccharides accompanied by the formation of aldehydes which cause a purple-red stain in reaction with Schiff's reagent (Vacek 1995). The presence of uronic acids may be the reason why a smaller amount of aldehydes which would react with Schiff's reagent is formed during staining. Another possible cause of weak staining, especially in the sample of guar gum, could be insufficient oxidation by periodic acid. Certain saccharides, particularly those of microbial nature, require oxidation with a more reactive form of acid (chromic acid) or what is known as double oxidation. The differing capability of oxidation by various oxidation agents could be used in connection with the precise measurement of colour for the differential diagnosis of the origin of a specific polysaccharide. The staining results are summarised in Table 3.

Table 3. PAS Calleja staining on selected hydrocolloids

Hydrocolloid	Resultant colour of PAS reaction	Intensity of staining
Carrageenan Lambda	pink-red	+++
Carrageenan Kappa	pink-red	+++
Carrageenan Iota	pink-red	+++
Potato starch	pink-red	+++
Xanthan gum	blue-purple	++
Guar gum	almost no staining	-
Gum tragacanth	almost no staining	-
Sodium alginate	blue-purple	

+++ extremely strong staining, ++ weak staining, + extremely weak staining, - not stained

Conclusions

Many additives are widely used today in the production of comminuted meat products. They differ in terms of their characteristic properties, production costs and, thereby, differing market prices. Hydrocolloids, whose detection using PAS Calleja staining was the subject of this work, are used for their functional properties as, for example, thickeners, while some enable the formation of gels. Hydrocolloids also help form a homogenous emulsion and also stabilise such emulsions, preventing the gradual separation of the emulsified components over time. These are, then, substances that modify the physical properties of foods such as viscosity and texture and the associated appearance of the food. This work focused on the possibilities of use of special PAS Calleja staining with oxidation by periodic acid in model meat products containing a 1% addition of selected hydrocolloids (carrageenans, potato starch, xanthan gum, sodium alginate and plant gums – gum tragacanth and guar gum). PAS Calleja staining was shown to be extremely suitable

for model meat products containing added Lambda XE 4405, Kappa XE 4066 and Iota XE 4248 carrageenans, with the added carrageenans being stained an extremely pronounced purple-pink colour. The same results were obtained with the use of this stain on potato starch. A poorer staining effect was obtained in samples containing plant gums (xanthan gum and gum tragacanth) and sodium alginate which was probably caused by the uronic acid content which causes insufficient aldehyde groups, which would react with Schiff's reagent, to be formed during staining.

References

Budig J and Mathauser P 2007: Technical and technological aspects of the production of parts of small meat products in the past and present. Maso **18**: 10-18

Cardoso MJ, Costa RR, Mano JF 2016: Marine origin polysaccharides in drug delivery systems. Marine drugs **14**: 34

Comaposada J, Gou P, Marcos B, Arnau J 2015: Physical properties of sodium alginate solutions and edible wet calcium alginate coatings. LWT-Food Science and Technology **64**: 212-219.

Černý L 2007: What about meat? 1st Ed. TeMi CZ. Velké Bílovice, 119p (In Czech)

Ingr I 2011: Meat production and processing. Brno: Mendelova univerzita, ISBN 978-80-7375-510-2

Eliášová M, Pospiech M, Tremlová B, Jandásek J, Kubíčková K 2012: The native and the modified starches in the meat products – detection of the raw materials using microscopy methods. Maso **23**: 30-34

Farzi M, Emam-Djomeh Z, Mohammadifar MAA 2013: Comparative study on the emulsifying properties of various species of gum tragacanth. Int J Bio Macromol **57**: 76-82

Focke H 2010: A wide variety of natural additives for the meat industry. Maso **21**: 34

Kadlec P, Melzoch K, Voldřich M 2012: Overview of traditional food production. Key Publishing, ISBN 978-80-7418-145-0

Kalina T and Váňa J 2010: Sines, algae, fungi, bryophytes and similar organisms in contemporary biology. Praha: Karolinum, 608 p. ISBN 978-80-246-1036-8

Karimi N and Mohammadifar MA 2014: Role of water soluble and water swellable fractions of gum tragacanth on stability and characteristic of model oil in water emulsion. Food Hydrocoll **37**: 124-133

Llewellyn BD 2013: PAS Reaction for 1-2 Glycols. In: StainsFile: The Internet Resource For Histotechnologists. Available at: http://stainsfile.info/StainsFile/stain/schiff/reaction-pas.htm

Luallen T 2004: Utilising starches in product development A. C. Eliasson (Ed.), Starch in food: Structure, function and applications, Woodhead Publishing, Cambridge

McClements D 2005: Food colloids: principles, practices, and techniques. 2nd Ed. Boca Raton: Royal Society of Chemistry. Special publication. Royal Society of Chemistry (Great Britain), 609 p. ISBN 08-493-2023-2

Murphy P 2000: Starch G. O. Phillips, P. A. Williams (Eds.), Handbook of food hydrocolloids, Woodhead Publishing, Cambridge, 65 p

Pérez S and Bertoft E 2010: The molecular structures of starch components and their contribution to the architecture of starch review. granules: A comprehensive Starch-Stärke **62**: 389-420

Pospiech M, Marcinčák S, Turek P 2016: Additives in meat products. Maso **4**: 12-27

Regulation (EC) No. 1333/2008 of the European Parliament and of the Council of 16th December 2008 on food additives. Official Journal OJ L 354, 2008, 31.12.2008, 16 p

Rather SA, Masoodi FA, Akhter R, Gani A, Wani SM, Malik AH 2015: Xanthan gum as a fat replacer in goshtaba-a traditional meat product of India: effects on quality and oxidative stability. J Food Sci Technol-Mysore **52**: 8104-8112

Rather SA. Masoodi FA, Akhter R, Gani A, Wani SM, Malik AH 2016: Effects of guar gum as fat replacer on some quality parameters of mutton goshtaba, a traditional Indian meat product. Small Rum Res **137**: 169-176

Rocha T, Felizardo SG, Jane J, Franco CML 2012: Effect of annealing on these micrystal line structure of normal and waxy corn starches. Food hydrocoll **29**: 93-99

Stephen AM 1995: Food polysaccharides and their applications **67**: CRC press

Šárka E, Smrčková P, Seilerová L 2013: Rezistentní a pomalu stravitelný škrob. Chemické listy **107**: 929-935 (In Czech)

Tarte 2009: Ingredients in meat products: properties, functionality and applications. Springer Science & Business Media, LLC. New York, USA. 419 p . ISBN 978-0-387-71326-7

Vacek Z 1995: Histology and histology technics. Brno: Institut pro další vzdělávání pracovníků ve zdravotnictví, 1995. ISBN 80-7013-202-7

Velíšek J and Hajšlová J 2009: Food chemistry I. 3rd Ed. Tábor: OSSIS. 602 p. ISBN 978-80-86659-15-2

Yu XL, Li XB, Xu XL, Zhou GH 2008: Coating with sodium alginate and its effects on the functional properties and structure of frozen pork. J Muscle Foods **19**: 333-351

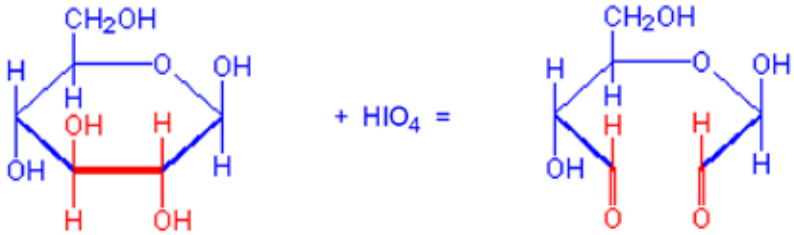
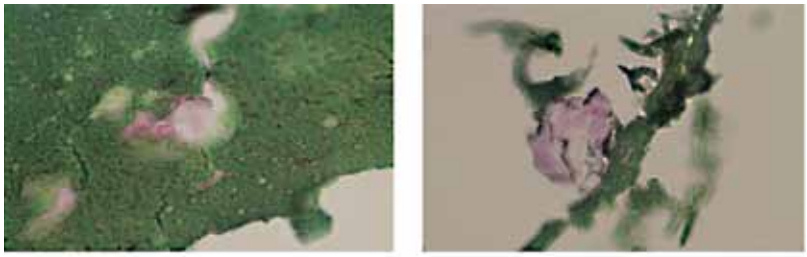
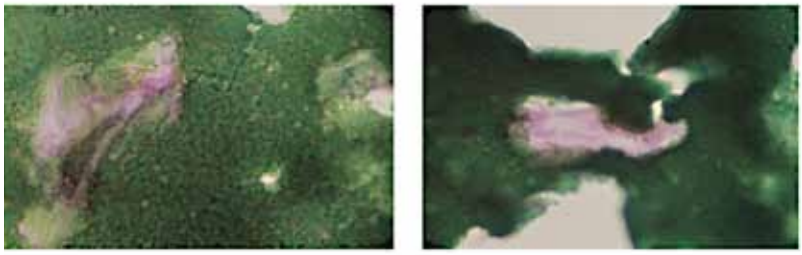


Fig. 1. The formation of aldehydes (L1ewellyn 2013)

A model meat product with the addition of the carrageenan Lambda XE 4405 (1%)



A model meat product with the addition of the carrageenan Kappa XE 4066 (1%)



A model meat product with the addition of the carrageenan Iota XE 4248 (1%)

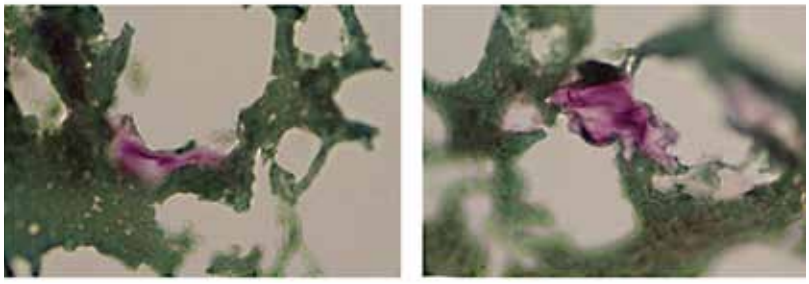


Fig 2. Staining results for carrageenans - 400 x

Plate V

A model meat product with the addition of potato starch (1%)

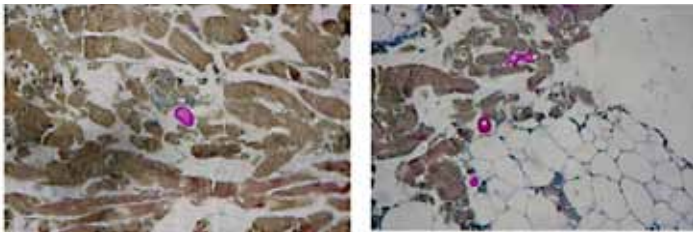
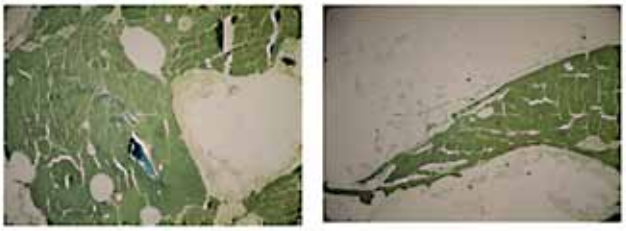


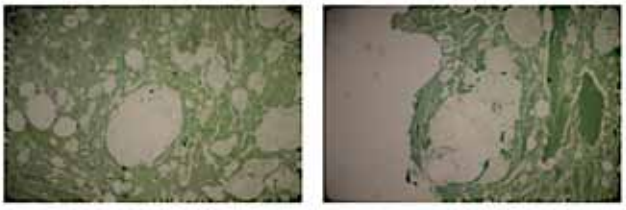
Fig 3. Staining results for starches - 100 x

A model meat product with the addition of xanthan gum (1%)

A model meat product with the addition of guar gum (1%)



A model meat product with the addition of gum tragacanth (1%)



A model meat product with the addition of sodium alginate (1%)

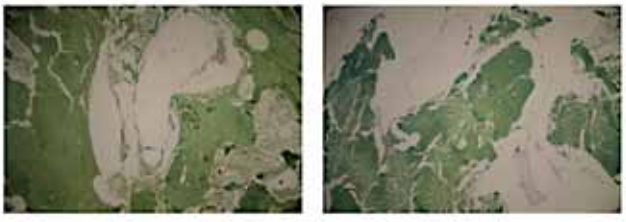


Fig 4. Staining results for plant gums, xanthan gum and sodium alginate - 100 x