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QUANTITATIVE DETECTION OF ADULTERATION OF VARIOUS PROCESSED MEAT PRODUCTS WITH SOYBEAN PROTEIN BASED ON DIFFERENT HISTOLOGICAL METHODS

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ABSTRACT

Egypt's meat producers produce a variety of meat brands that used soybean proteins because of high meat pricing. Allergens have been discovered in products that were not declared allergen-free. A total of 540 samples were obtained at random from various food supermarkets in Sohag City. Using haematoxylin and eosin and various histochemical dyes, light microscopy was utilized to identify the structural properties of the soybeans. The structure of all components was determined using fluorescent microscopy and acridine orange dye. Soybeans were discovered in meat samples and the cotyledon's vascular tissue and parenchymal cells rich in pectin confirmed this discovery by immunohistochemistry techniques. After immunohistochemical confirmation with an antirabbit soybean marker, the proportion of soybean was determined using histological procedures and image analysis. The percentage of soybeans in various meat products, including minced meat from two quality levels, sausages from two quality levels, raw kofta, and beef burgers from two grades, as well as chicken and luncheon meats, was 85%, 90%, 64%, 76%, 80%, 65%, 97%, 82%, and 69%, respectively. Soybean percentages in the examined samples did not fulfill Egyptian standards for soya percentages, which is approximately 10%, and this may affect consumers' health. In conclusion, all tested meat samples contained a high percentage of soya adulteration, necessitating increased supervisory to reduce meat fraud.

Keywords: Immunohistochemical stain; scanning electron microscope and fluorescent microscope; light microscopy; soybean; meat products.

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INTRODUCTION

Meat is a main source of dietary protein and is considered a favourable food because of the delicious taste and flavour, rendering meat widely consumed in developing countries. To cover the high requirements of meat production and achieve balance with high economic value, adulteration of meat products during manufacture has become common, particularly for minced and comminated meat products. Meat fraud is widely performed using different types of meat species or vegetable proteins (Belloque *et al.,* 2002; Fajardo *et al.*, 2008; Pascoal *et al.,* 2004). Other forms of meat fraud include inaccurate labelling of meat products and the use of undeclared substances, including allergens, besides the replacement of meat by commercially cheaper species. Aside from financial concerns, consumers are concerned about food quality based on their health, nutrition, religion, and lifestyle. All these criteria necessitate increased supervisory responsibilities to reduce meat fraud and protect consumer rights. Consequently, evaluating and validating meat product components is crucial for the food industry (Meyer *et al.,* 1996).

Unlabelled vegetable proteins are commonly used in meat products. Hence, the European Union (EU) instituted regulations on food labelling concerning 14 classes of allergenic components, including soybeans, that must be listed on the labels of food items [Directive 2007/68/EC, Directive 2007/68/EC; Regulation (EU) No 1169, 1169/2011] (Meyer *et al.,* 1996). Soybeans are frequently applied as a vegetable protein in the meat industry because of their technological features, such as emulsifying characteristics, gelling tendency, textural benefit, and water binding capacity or moistening capacity (Muhamad *et al.,* 2016). Moreover, soybeans provide a well-balanced amino acid composition (Friedman & Brandon, 2001). Soybeans comprise approximately 70% of the total soy protein, which is considered a complete food and contains nine essential amino acids (Singh *et al.,* 2008). Additionally, soybeans have a low level of cholesterol (Snyder & Wilson, 2003) and no lactose (Hendricks & Guo, 2021). For these reasons, soybean proteins are the most common additives to meat products (Belloque *et al.,* 2002). Moreover, soybeans improve the hygienic quality of meat products. Minced beef mixed with soybeans is one of the meat products widely consumed in Egypt.

Soy protein has a protective function against diabetes mellitus, plasma cholesterol, cancer, and obesity. It also has been shown to improve renal, cardiovascular, and gastrointestinal disorders (Bhathena & Velasquez, 2003). However, others suggested that soybeans had harmful effects, including hormone abnormalities, carcinogenicity, and organ toxicity (Sukalingam *et al.,* 2015).

Some countries have standards and/or restrictions on the amount of added vegetable proteins in various meat products. In Portugal, for example, it has been suggested that Frankfurter sausages should contain no more than 5% vegetable proteins as an optional ingredient (NP, 2006). In the United States, a maximum of 3.5% soybean flour and 2% soybean protein isolate are allowed in sausages (Belloque *et al.*, 2002). In Spain, a maximum of 3% soybean protein isolate is allowed (dry basis) (Castro *et al.,* 2007), and in Brazil, a maximum of 0.04% soybean protein can be used in certain meat products, including “hot dog” sausages and hamburgers (Brod & Arisi, 2007). In these circumstances, the estimation of additional proteins is required to ensure compliance with label declarations and current regulations.

Soy protein determination in meat products is frequently linked to concerns with components and matrices. Soybeans have been detected and quantified using a variety of methods such as immunological assays and electrophoretic and chromatographic

procedures ((Belloque *et al.,* 2002; Castro *et al.,* 2007; Koppelman *et al.,* 2019; Macedo-Silva *et al.,* 2001). Other techniques are applied to detect soy protein in meat products including a microscopic method that uses histochemical techniques and image analysis (Pospiech *et al.,* 2011; Sadeghinezhad *et al.,* 2016) immuno-histochemistry (Pospiech *et al.,* 2011), immunofluorescence (Llewellyn & Flaherty, 2006), an ELIZA (Sánchez-Martínez *et al.,* 2009), the HPLC method (Criado *et al.,* 2005), and gold nanoparticles (Sánchez-Martínez *et al.,* 2009). Besides molecular techniques, PCR can be used to quickly identify native or modified soybeans (Abd El-Nasser *et al.,* 2010). Additionally, the TaqMan real-time PCR (Soares *et al.,* 2014) is used to analyse soybean proteins.

The goals of this study were to determine the extent to which various meat items in Sohag food markets were adulterated with soybeans. Besides scanning electron microscopy examinations, we used simple histological procedures to identify distinct soya structures and validate soya marker detection.

MATERIAL AND METHODS

Sample collections

540 samples were collected from nine distinct meat products (minced meat of two different qualities, raw kofta, sausages of two different qualities, and beef burger from two different qualities, chicken luncheon and meat luncheon of the same brand). In factories, minced meat samples were mechanically processed and completely blended. As a result, meat samples were used to reflect the real components of meat products. From food stores in Sohag city, 60 random samples of each product were obtained. There was no mention of soybean on any of the sample labels for these products.

30 samples were utilized for light microscopy and another 30 samples were used for scanning electron microscopy. From each sample, we select a tiny specimen for the frozen cutter section and fix it in formal calcium.

Frist technique: Light microscopic techniques

1-Fixation and processing of samples for paraffin embedding blocks, staining with different histochemical stain and examined by light microscope

From each specimen, three samples of $1cm^3$ were collected from three separate parts and fixed in Serra's fixative (100 percent ethanol, 40 percent formaldehyde, and glacial acetic acid in proportions 60: 30: 10) (Massoud *et al.,* 2021). Therefore, 90 paraffin blocks were used. The time of fixation was about 24 hours at 4°C. The following procedures were used to prepare the samples for light microscopic examination: The fixed samples were carefully washed with 70% ethanol alcohol for three days. The samples were dehydrated in increasing concentrations of ethanol alcohol (80% for one hour, 90% for one hour, 100% I and 100% II for half an hour each). After that, they were cleared for two days in each of methyl benzoate I and II. Each paraffin paraplast change, paraffin I, paraffin II, and paraffin III, was embedded for one hour. A (Richert Leica RM 2125 Microtome, Germany) was used to cut serial sections of 5-7 µm from each paraffin-embedded block and mount them on glass slides. For dryness, the sections were maintained in a 40°C incubator.

1. 1 Stains used in paraffin sections

Several traditional stains were used to determine the soybean structure in this study. For general histological examinations, Harris Hematoxylin-eosins (HE) (Harris, 1900) were employed; in addition, the following staining techniques were utilized: Trichrome Crossomon's, Van Gieson, Verhoeff's-Van Gieson stain (VVG); Alcian blue pH 2.5 and periodic acid-Schiff reaction (PAS), and the bromophenol blue. All staining protocols were following (Suvarna *et al.,* 2013)

1.2 Histochemistry on frozen sections and examined by light microscopy (ATPase enzyme histochemistry)

We take half-cm^3 samples of each sample product for frozen sections. After fixation in formal calcium, materials were soaked overnight in Optimal cutting temperature compound (OCT) in the fridge at 4 °C and then stored at -20 °C for further use in cryosection (Abou-Elhamd *et al.,* 2015). They were stained with ATPase enzyme histochemistry as indicated by (Suvarna *et al.,* 2013)

Second technique: Fluorescent microscopy

Use Acridine orange dye to stain paraffin sections and examined by Epifloroscent microscope. The procedure is based on that of Hoff and colleagues (Hoff *et al.,* 1985) modified by (Abd-Elhafeez *et al.,* 2023)

Third technique: scanning electron microscopy

1-Sample processing

Three representative specimens from each sample were washed three times in phosphate buffer pH 7.2 and then fixed in a Karnovsky fixative (Karnovsky, 1965) at 4°C for 24 hours for scanning microscopic inspection and processed according to (Abd-Elhafeez *et al.,* 2016). The samples were coated with gold using a JEOL -1100 E-ion-sputtering device and studied at KV10 at Assiut University's Electron Microscopy Unit with a JEOL scanning electron microscope (JSM – 5400 LV). Scanning electron microscopy was utilized to analyse unfixed soya seed grinding, which was then coated with gold using a JEOL -1100 E-ion-sputtering equipment.

2- Digitally coloured scanning electron microscopic images using Photoshop CS6

We used the Photoshop CS6 program to digitally colour the scanned electron microscopic images to discern the structure of soybean on the same electron microscopic picture. Different authors (Abd-Elhafeez *et al.,* 2022; Abdel-Maguid *et al.,* 2019; Emeish *et al.,* 2023; Soliman *et al.,* 2023) used the same techniques .

Fourth technique:

1-Immunohistochemical staining

Antigen localization was achieved using mouse antirabbit antibody against matrix combined with the avidin–biotin complex (ABC) technique (Hsu *et al.,* 1981). We used the reagent of the Ultra-Vision Detection System [Anti-Polyvalent, HRP/DAB (ready to use, TP-015-HD: 15 mL Hydrogen Peroxide Block (TA-015-HP), 15 mL Ultra V Block (TA-015-UB), 15 mL Biotinylated Goat Anti-Polyvalent (TP-015-BN), 15 mL Streptavidin Peroxidase (TS-015-HR), 15 mL 3,3′-diaminobenzidine (DAB) Plus Substrate (TA-015-HSX), 1 mL DAB Plus Chromogen (TA-001-HCX), Thermo Fischer Scientific TP-015HD, UK Lab Vision Corporation; USA).] according to the manufacturer's instructions. The procedure, according to the description of Alnasser (Alnasser *et al.,* 2023), was as follows: paraffin sections of (5 μm) were dewaxed by xylene, rehydrated by descending grades of alcohol, and rinsed by PBS at a pH of 7.4 (3 times for 5 min). Endogenous peroxidase was suppressed by using a hydrogen peroxide block at room temperature. The sections were washed by pH 7.4 phosphate buffer solution (PBS) for an additional 10 min. Afterwards, to enhance antigen retrieval; the slides were treated with 10 mm pH 6.0 sodium citrate buffer at a temperature of 95–98°C in a water bath for 20 min. The sections were cooled for 20 min at room temperature and subsequently were washed in PBS, 3 times for 5 min). Blocking non-specific background staining was performed by using Ultra V block for 5-10 min at room temperature. The primary antibody (rabbit anti-soya, Sigma S28519, Germany) diluted in blocking solution (1:500) was applied to sections overnight at 4^{o}c. Sections were washed using PBS (3 times for 5 min). The incubation processes were carried out in a humid chamber. The biotinylated secondary antibody, goat Anti-Polyvalent, was applied for 10 min at room temperature. Sections were washed by PBS

(3 times for 5 min) and subsequently incubated for 10 min at room temperature with the streptavidin– peroxidase complex. Visualization of the bound antibodies was performed using one drop of DAB plus chromogen to 2 mL of DAB plus substrate. The mixture was applied and incubated at room temperature for 5 min. The sections were dehydrated using ethanol alcohol 90% and (100% I and II), cleared in xylene and covered by DPX. The Immunostained sections were examined under a Leitz Dialux 20 Microscope and images were captured using Canon digital camera (Candison Power shot A95) attached to the microscope.

Buffers constituents used in immunohistochemistry procedure: we used pH 7.4 phosphate buffer solution and 10 mm pH 6.0 sodium citrate buffer.

PH 7.4 phosphate buffer solution: 10 mm pH 6.0 sodium citrate buffer: Solution A: Na2HPO4 2H2O 17.02 g + Distilled water 600 ml. Solution B: NaH2PO4 H2 6 g +Distilled water 200 ml, using solution composed of Solution A 580 ml + Solution B 219 ml

10 mm pH 6.0 sodium citrate buffer: Solution A: Citrate C6H8O7·H2O 21 g + one litter of Distilled water. Solution B: Sodium citrate 41 g + one litter of distilled water. Using solution composed of 9 mL of Solution A + 41 mL of Solution B ml + 500 mL of Distilled water

The quantification of soybean content percentage according to immunostaining section and histochemical stains

The number of samples examined, 3 representatives' areas from 30 samples, was sufficient to assess soybean adulteration. Several studies used histological analysis to determine the content of plants in meat products (Pospiech *et al.,* 2011; Sadeghinezhad *et al.,* 2015). Immunohisto-chemical methods have been identified as the most appropriate for verifying the results.

The amount of soya in the meat products was carefully measured using Fiji software (Image J) (http:// fiji.sc/Fiji). For quantification of soya bean protein, the immunostained particles from three portions from various locations of each section were counted at magnification X4 and calculate as the area coverage percentage of immuno-stained particles according to (Abd-Eldayem *et al.,* 2022).

For quantification of immunohistochemal images using image J and how to remove background in image J, follow the provided link: **https://www.google.com/search?q=quantification+of+immunohistochemistry+images+using+imagej+%7C+how+to+remove+background+in+imagej&rlz=1C1GCEA_enEG992EG992&oq=q&aqs=chrome.1.69i57j35i39j0i131i433i512j46i199i291i433i512j0i433i512l2j46i433i512j0i512j0i131i433i512j46i131i199i433i465i512.2237j0j15&sourceid=chrome&ie=UTF-8**

RESULTS

I. First section of results: Identification of soybean structures, without any mixing with meat products, by applying the techniques of electron, scanning, fluorescent and light microscopy methods

The purpose of this section including all the supplemental Figures (Suppl 1-6) is to describe the natural shape of soy structure without any mixing with meat products. The description will make it easier to identify any soy structure in beef products using various histological techniques or scanning electron microscopy.

II. Second sections of result:

Soybeans were identified in various meat preparations using various techniques (light, Fluorescent, Scanning electron microscope, and immunohistochemistry).

1-Minced meat of different qualities

a-Soybean structures were discovered in high-quality minced meat: Fig. 7A- C.

b-Soybean structures were discovered in a low-quality minced meat sample: Fig. 7D-F.

2-Beef burger with two different qualities
a-Soybean structures were discovered in a high-quality beef burger sample: Fig. 8A-P.
b-Soybean structures were discovered in a low-quality beef burger sample: Fig. 9A–I.

3-Sausage of two different qualities
a- Soybean structures were discovered in a high-quality beef sausage sample: Fig. 10
b- Soybean structures were discovered in the beef sausage sample of low-quality: Fig. 11.

4- Soybean structures were discovered in raw kofta samples: Fig. 12.

5-Soybean structures were discovered in chicken luncheon samples: Fig. 13A-I

6-Soybean structures were found in luncheon meat: Fig. 13J–R.

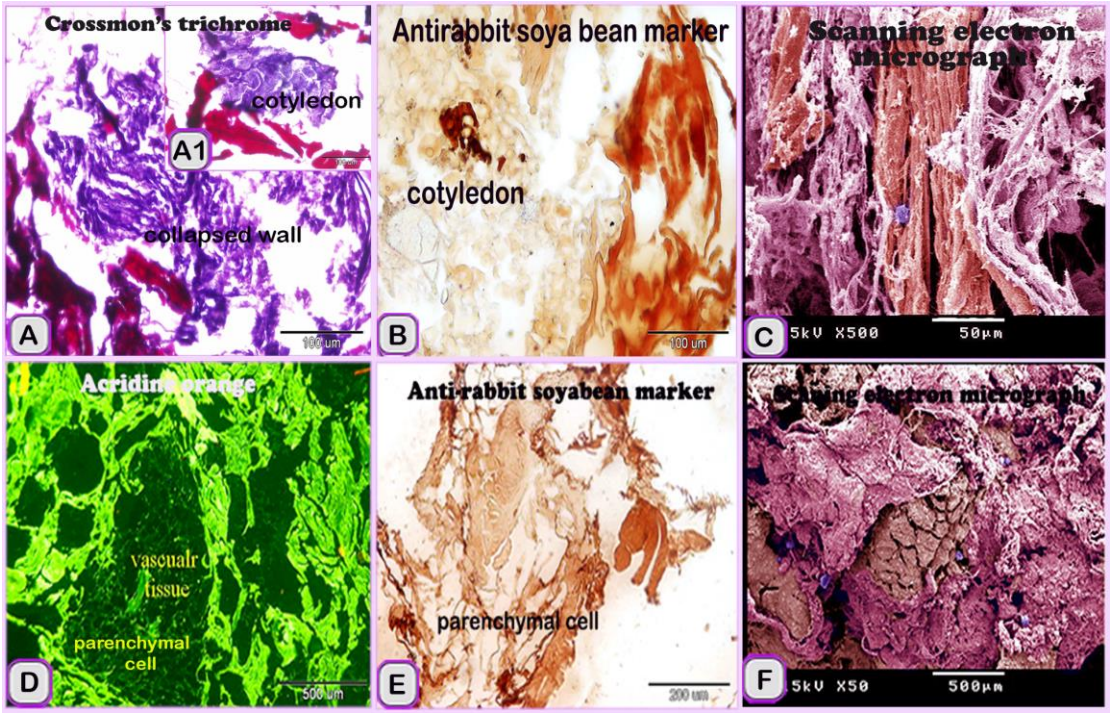

Fig 7: Soybean was discovered in paraffin sections and scanned samples of high-quality (A–C) and low-quality (D–F) minced beef.

A: Cotyledons of soybeans identified by the collapsed wall of parenchymal cells. B: The cell walls of parenchymal cells and cotyledons had collapsed and were positive for the soybean marker. C: Scanned samples indicated collapsed parenchymal cells (pale pink colour). D: Soybean cotyledons were distinguished by parenchymal cells and vascular tissue. E: Soybean parenchymal cells were shown to be positive for the soybean marker. F: Collapsed parenchymal cells (pink colour) within skeletal muscle fibers (brown colour).

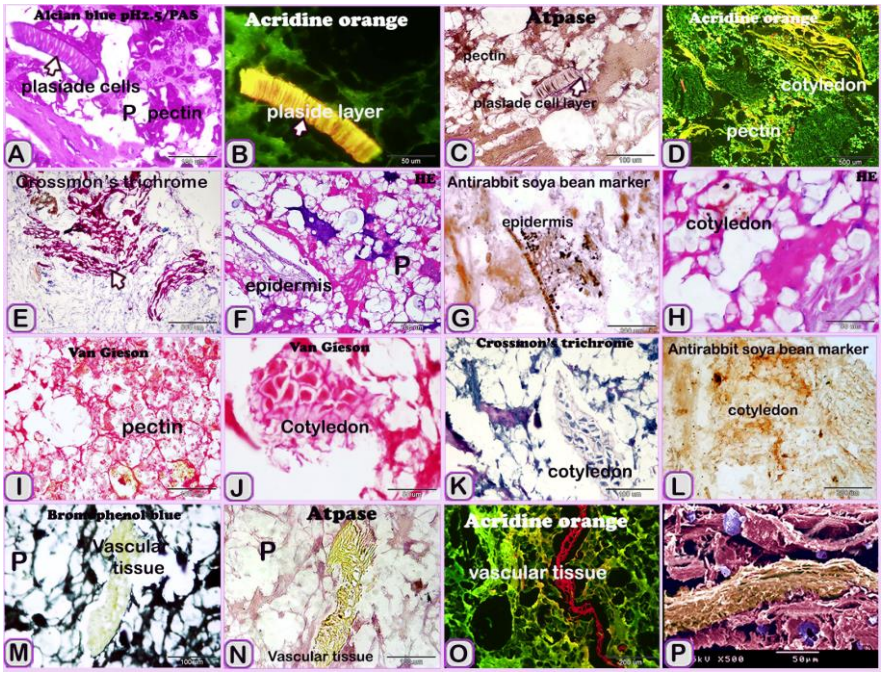

Fig 8: Soybeans were discovered in high-quality paraffin sections of beef burgers and scanned electron micrographs.

A: Cotyledons of soybeans were indicated by pectin-rich parenchymal cells. The palisade layer (arrows) indicated soybean husks and pectin stained positive for PAS. B: Cotyledons of soybeans were identified by observation of parenchymal cells. The palisade layer (arrows) indicated the husk. C, I: Parenchymal cells and palisade layers exhibited strong ATPase activity. D: Soybean cotyledons had pectin-rich parenchymal cells. E: Crossomon's trichrome was used to stain the collapsed walls of parenchymal cells red (arrows). F: Soybean cotyledon epidermis. G: The epidermis of the cotyledon was positive for an antirabbit soybean marker. H: HE was used to identifying soybean cotyledon parenchymal cells. I–K: Van Gieson stain stained the pectin-rich parenchymal cells red and Crossomon's stain stained them green. L: Parenchymal cells were positive for the antirabbit soybean marker. M, O: Cotyledons were identified by the vascular tissue (V) and the parenchymal cells (p). N: The vascular tissue exhibited weak ATPase activity. P: Soybeans within the scanned meat sausage showed minced and collapsed parenchymal cells (violet colour) of soybean cotyledons and husks (brown colour).

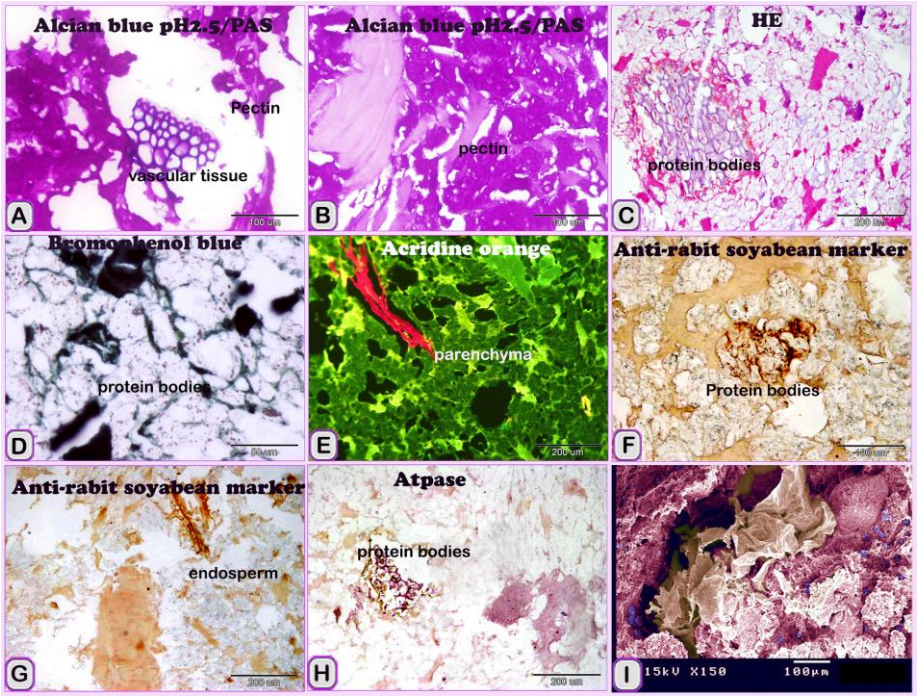

Fig 9: Soybeans were found in paraffin sections and scanned electron micrographs of beef burgers of low-quality.

A, B: Pectin-rich parenchymal cells and the vascular tissue were recognized as from soybean cotyledons': HE stains of acidophilic protein-rich parenchymal cells. D: Protein bodies stained positive for bromophenol blue. E: Besides identified parenchyma, the soybean endosperm was coloured red with an acridine orange stain. F: Soybean cotyledons were recognized with an antirabbit soybean marker as parenchymal cells that contained stained positive protein bodies were observed. G: The soybean endosperm demonstrated a higher affinity for the soybean marker than parenchymal cells. H: Protein bodies exhibited strong ATPase activity. I: A scanned sample showed the parenchymal cells (pink) contained pectin (blue) and husks of soybeans (yellow).

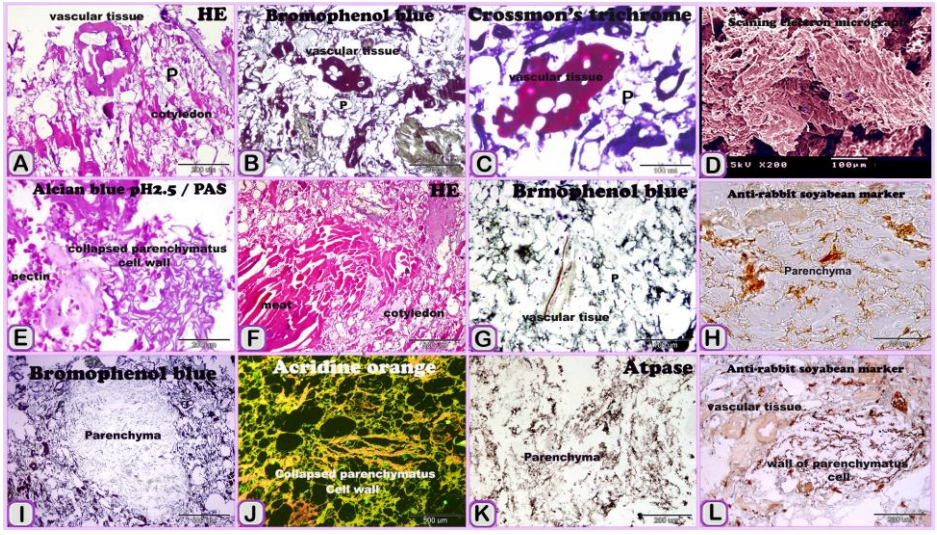

Fig 10: Soybeans were discovered in paraffin sections and scanned electron micrographs of high-quality beef sausage

A–C, F, G, I: Cotyledons of soybeans identified by the observation of parenchymal cells and vascular tissue, D: A scanned sample showed collapsed parenchymal cells (brown). E: Cotyledons of soybeans were identified by the pectin that was liberated from the minced parenchymal cells. H: Cotyledon of soybeans were identified by the parenchymal cells, which exhibited strong positivity for an antirabbit soybean marker. J: Cotyledons of soybeans were recognized via the observation of the wall of the collapsed minced parenchymal cells. K: Parenchymal cells of the cotyledon exhibited strong ATPase activity. L: Parenchymal cells of the cotyledon exhibited strong immunostaining affinity and the vascular tissue exhibited less immunostaining affinity by an antirabbit soybean marker.

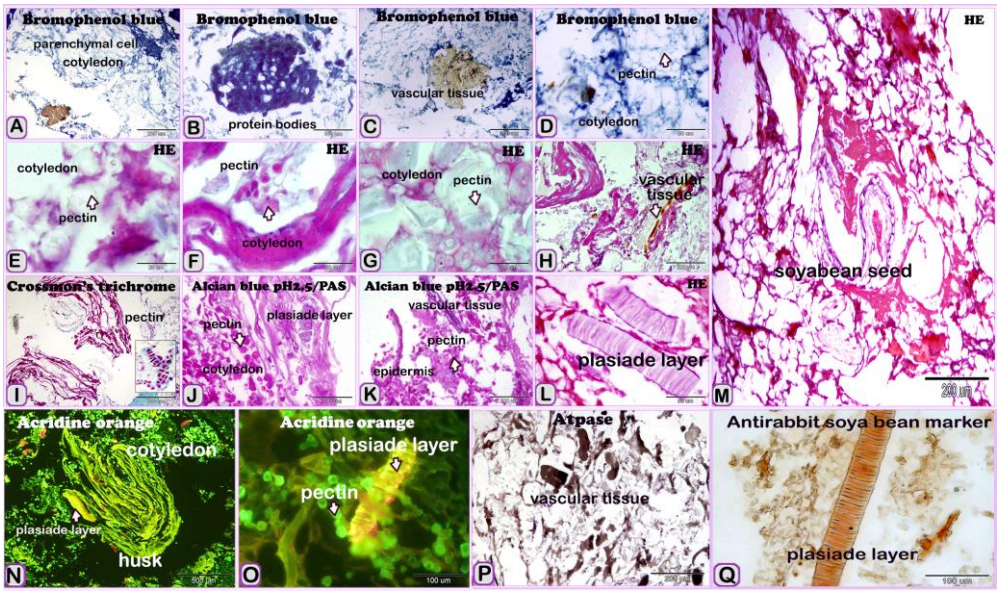

Fig 11: Soybeans were discovered in paraffin sections and scanned electron micrographs of low-quality beef sausage.

A: Meat sample that contained soybeans, which was identified by observing the parenchymal cells of the cotyledons. B: Parenchymal cells of the cotyledon were rich in protein bodies, which were positive for bromophenol blue. C: The vascular tissue and parenchymal cells indicated cotyledons. D, E, G: Parenchymal cells of the cotyledon were rich in pectin (arrows). Note the vascular tissue was located between the parenchymal cells. F: Parenchymal cells of the cotyledons were rich in protein bodies. H: Cotyledons were identified by the vascular tissues. I: The pectin-rich parenchymal cells stained red by Crossomon's trichrome indicated cotyledons. J–L: The palisade layer indicated soybean husks. The epidermis, pectin-rich parenchymal cells (arrows), and the vascular tissue distinguished cotyledons. M: Seeds of soybeans. N, O: Cotyledons identified by pectin (arrow) that was liberated from the parenchymal cells during mincing. O: Palisade layer (arrow) of soybeans. P: Parenchymal cells and the vascular tissue exhibited ATPase activity. Q: The palisade layer was positive for the soybean marker.

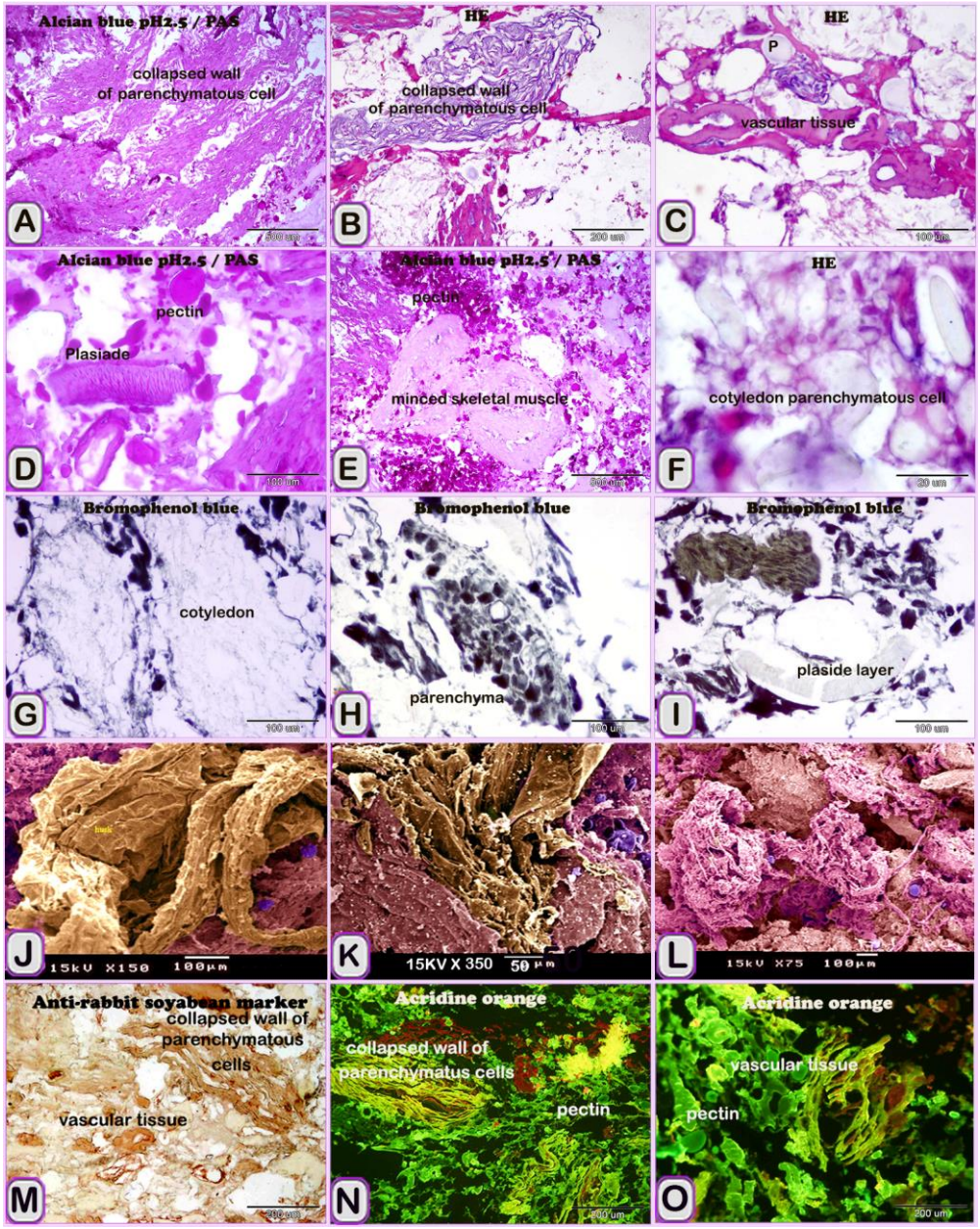

Fig 12: Soybeans were found in paraffin sections and scanned samples of raw kofta

A, B, E: Cotyledons of soybeans were identified by a collapsed wall of parenchymal cells. The cell walls of parenchymal cells and released pectin were stained positive for PAS following mincing. C, D: Cotyledons of soybeans were identified by observing vascular tissue and pectin (P). Note positive staining of pectin and the palisade cell layer was observed with combined Alcian blue pH 2.5/PAS stain. F: Cotyledons of soybeans were identified by observing pectin. G, H: Cotyledons of soybeans were identified by observing parenchymal cells. I: The palisade layer was identified in soybean husks. J–L: Scanned meat samples with the husks of soybeans (yellow) and collapsed parenchymal cells (violet) that contained pectin (blue). M: Collapsed parenchymal cells and vascular tissue were positive for a soybean marker. N, O: Collapsed parenchymal cells, vascular tissue, and pectin were recognized with acridine orange stain.

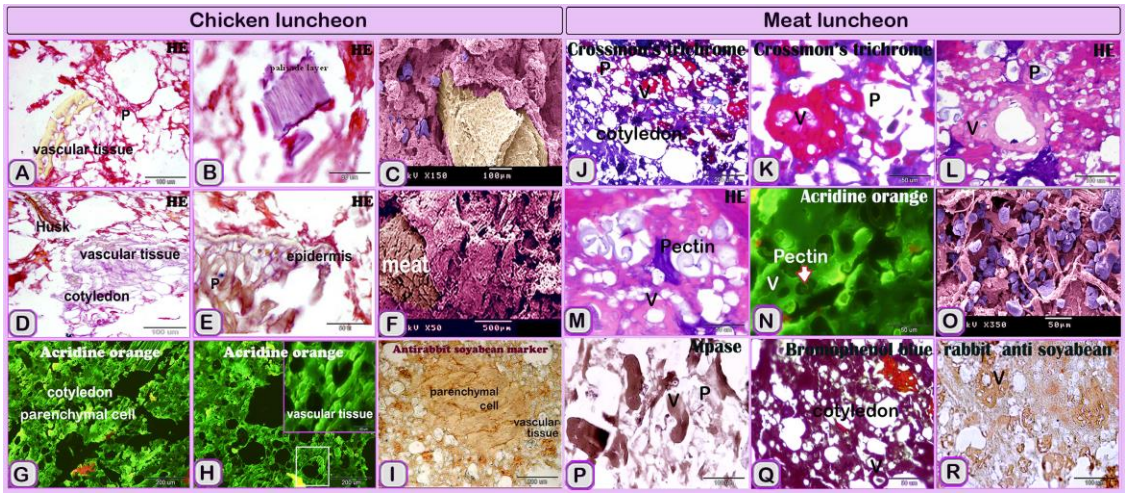

Fig 13: Soybeans were found in paraffin scanned chicken (A–I) and luncheon meat (J–R) samples.

A: The vascular tissue and the parenchymal cell (p) indicated cotyledons of soybeans. B: Palisade layer of soybean husks. C: The epidermis and pectin (blue)-rich parenchymal cells were recognized within the scanned luncheon chicken samples; soybean cotyledons (pink) were also identified. D: Cotyledons of soybeans were recognized by observing husks and vascular tissues. E: The epidermis and parenchymal cells indicated cotyledons of soybeans. F: Scanned chicken luncheon meat samples had husks (pink) of soybeans. G, H: Acridine orange staining indicated cotyledon pectin and vascular tissue. I: Cotyledon collapsed parenchymal cells were positive with soybean a marker. J, K: The parenchymal cells (p) and vascular tissues (V) stained red by Crossomon's trichrome stain indicated cotyledons. L, M: Cotyledons were identified by the acidophilic vascular tissues (V) and the parenchymal cells that contained basophilic pectin (p) according to HE is staining. N: The vascular tissues (V) and parenchymal cells, which contained pectin (p) according to acridine orange staining, identified cotyledons. O: Scanned luncheon meat samples had soybean cotyledons (pink) identified by pectin blue-rich parenchymal cells. P: Vascular tissue (V) and parenchymal cells exhibited ATPase activity. Q: Cotyledons were stained with bromophenol stain. R: An immunohistochemical marker for soybeans detected the vascular tissue (V) and the parenchymal cells of the cotyledon.

III. The third section of results: Quantification of the soybean percentage in the meat products:

The percentages of soybeans in various meat products, including minced meat of two quality levels, sausages of two quality levels, raw kofta, and beef burgers of two grades, as well as chicken and luncheon meats, were about 85%, 90%, 64%, 76%, 80%, 65%, 97%, 82%, and 69%, respectively.

DISCUSSION

Food product quality and the authenticity and reliability of food commodities and safety need immediate confirmation to assure consumers. This necessity is becoming increasingly significant as a result of expanding global demands to know the origins of food products, identification of hazards associated with the use of improper food items on human health, and other considerations (Opara, 2003).

Validation is characterized by the ability to identify animal species and products at various phases of the food supply chain, from production to distribution, according to European Commission Law 178/2002 (Murugaiah *et al.*, 2009). Histochemical analyses were applied to assess adulteration

by soybeans and addressed the extent of meat product safety. The percentage of soybeans in various meat products, including minced meat from two quality levels, sausages from two quality levels, raw kofta, and beef burgers from two grades, as well as chicken and luncheon meat was 85%, 90%, 64%, 76%, 80%, 65%, 97%, 82%, and 69%, respectively. These results revealed that all meat products are unsafe for human consumption.

The percentage of the ingredients, especially the hazardous components, should not exceed the permissible limit during the manufacture of meat products. The Codex–Aliment Arius Commission, the World Health Organization, the Food and Agriculture Organization, and the European Commission recently announced a list of 12 allergy categories on the basis of their occurrence and severity, and food packaging must mention their names on the labels. Soybeans are included in this list (WHO, 2001) because they can trigger a variety of allergies even in low concentrations (Galan *et al.*, 2011).

Labelling of meat products has restrictions to assure the safety of the meat product. If the proportion of plant protein to meat species in products comprising red meat, and chicken, is less than or equal to 1:13, the name of the plant protein, as well as the product's name, should be listed on the ingredients label (Hui, 2007). Soybean percentages in the examined samples did not fulfil Egyptian standards for soya percentages of approximately 10%, and this can affect consumers' health.

Hamburgers are one of the most extensively consumed beef products throughout the world. A hamburger in Iran is described as minced red meat from Halal sources, mainly beef, to which other components, such as plant protein (soybean and gluten), oil, spices, filling and binding materials, salt, and fragrant herbs, are added. Factory-made hamburgers are categorized into three types according to the National Iranian Standards: ordinary burgers, premium burgers, and so on. Burgers typically contain at least 30% red meat, as well as a specified percentage of plant proteins and other approved components. This category of premium burgers contains 60%–74% beef and other authorized components, but no soybean protein. Another type of premium burger has 75%–95% beef, no soybean protein, and other components that are allowed (Institute of Standards). Only beef is permissible in the manufacture of factory-made hamburgers, according to manufacturing permits provided by the Ministry of Health and Medical Education (Hosseini *et al.*, 2009).

(Jahed Khaniki & Rokni, 2004) used a histochemical technique to identify soybean tissue in frozen raw Iranian burgers, and they detected soybean tissue. Improper food labelling suggests a different form of consumer-harming adulteration, improper labelling, conversely, may leave allergens unmentioned, putting consumers who are sensitive or allergic to them at risk (Pascoal *et al.*, 2004). We discovered soybean percentages in two types of minced meat of variable grade, approximately 85% and 90%. (Ahmed & Takwa, 2010) enhanced the microbiological meat quality and prolonged the shelf life of the minced beef to 7 days of retail displayed at 4°C by adding 0.3% and 0.5% soya extract. We discovered two types of soybean percentages, i.e., approximately 65% and 97%, in sausages of variable grades. There are guidelines and/or restrictions in some countries regarding the amount of added vegetable proteins in various meat products.

In luncheon meats, the soya results revealed approximately 82% and 96% of chicken luncheon meats and beef luncheon meats of the same brand. Our findings are consistent with those of (Malak *et al.*, 2020).

A previous study used PCR for the detection of soybeans in different meat products including the following: minced meat, raw kofta, sausages, and beef burgers with native soybean. They had adulteration rates of

50%, 72.7%, 75%, and 100%, respectively. The authors found that the minced meat percentage was approximately 58%, whereas the medium quality minced meat percentage was approximately 90%. The high-quality percent in our analysis may not have been included in Abd El-Nasser *et al.* (2010), whereas the medium quality percent in our study may have been the same brand as in. In our study, the percentage of sausage found by histological methods was approximately 76% in low-quality brands and approximately 64% in high-quality brands, which was similar to the proportion found using PCR by Abd El-Nasser *et al.* (2010). The raw kofta percentage of soybean in our study was almost 80%, which was similar to the result of Abd El-Nasser *et al.* (2010). The differences may be related to brand and preparation technique variances. In our experiment, the percentage of soybean in a low-quality brand beef burger was approximately 97%, which is like the finding of Abd El-Nasser *et al.* (2010), which was approximately 100%. The discrepancy probably results from the use of different approaches. The percentage of a high-quality soybean was roughly 65%. The percentage discrepancy from Abd El-Nasser *et al.* (2010) could be attributed to brand variances.

(Sukalingam *et al.*, 2015) summarized the risk of increased soybean quantity on the basis of several experimental studies. Among soybean proteins, genistein, an active ingredient, has an estrogenic action, which stimulates the proliferation of estrogenic receptors in breast cancer cells (Taylor *et al.*, 2009). Humans absorb a lot of genistein, a soybean isoflavone. Its estrogenic activity, however, has a negative impact on the development of the male reproductive system, and it diminishes sperm production (Lee *et al.*, 2004). In animal studies, genistein produced hyperplasia of Leydig cells in the testis as well as causing severe damage to the epididymis. Furthermore, during the juvenile stage, there was a decrease in sperm counts and an increase in sperm motility (Ekor *et al.*, 2010). Carcinogenic effects of soybeans may extend to the pancreas (Liener, 1996) and thyroid glands (M., 2003). Soybeans may also cause nephrotoxicity (Jin *et al.*, 2009; Zhao *et al.*, 2005) and hepatotoxicity (Senthilkumaran *et al.*, 2013; Wiwanitkit, 2012).

A large amount of soybean protein contains phytoestrogens such as zenistein, biochalin A, and daidzein. The majority of phytoestrogens are endocrine disruptive substances that interfere with hormone and reproductive system activities (Kwack *et al.*, 2009), and they have an impact on sexual development in terms of puberty timing, oestrogen cycle impairment, ovary functioning, and pituitary and hypothalamic dysfunctions according to several investigations (Guo *et al.*, 2005). Furthermore, it has been stated that increased soybean protein usage resulted in detrimental estrogenic and goitrogenic effects (Xiao *et al.*, 2004). Consuming soybean protein can cause thyroid hormone functioning and iodine utilization to decrease over time (Huang *et al.*, 2005). Daidzein, a powerful thyroid inhibitor, is found in soybean proteins. Soybean proteins work as an antithyroid agent, lowering iodine absorption (Jeffrye & Jones, 2005; Xiao, 2008). Several researchers have concluded that high consumption of soybean proteins causes thyroid suppression and goitre (Divi *et al.*, 1997) in iodine-deficient animals and new-borns (Jabbar *et al.*, 1997) based on thyroid function tests. In iodine-deficient rats, soybean proteins also promote the development of thyroid hyperplasia (Son *et al.*, 2001).

CONCLUSIONS

This study emphasized the significance of meat inspection programs to strengthen food labelling compliance, eliminate fraudulent practices, and protect the health of allergic consumers. The described methodology was shown to be a valuable tool for authentication/control, enabling

identification and quantification of soybeans in complex meals like processed meat products. This study described the soya structure within different meat products using routine histological stains, scanning electron microscopy, and determined the soya percentage according to immunohistochemistry for soybean protein identification. In the case of raw minced meat, the processing of meat samples makes it difficult to gather sufficient data for proper results and interpretation. Vice versa, SEM, on the other hand, is a suitable approach for examining a big area and a wide range of samples in order to produce sufficient data. We believe that for all meat products involved in this study, the applied immunohistochemistry procedures after standard histological techniques will be helpful in all labs.

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DATA AVAILABILITY

All data collected or analyzed during this investigation are available upon request from the corresponding author.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

The research study was designed by H. H. A., S. A. S, D.S.A., and R.S. Z.
H. H. A., S. A. S, A. M. E., D.S.A., and M. A. A., A. A contributes to the discussion. D. S. A., and R.S. Contributed to the idea of the work. H. H. A., D. S. A, and R.S. Z. funded immunohistochemical staining and scanning electron microscopy. H. H. A. and S. A. S, funded sample processing, histochemical and, Acridine orange, fluorescent stain. H. H. A. performed all the practical work, including sample processing, histochemical and immunohistochemical staining, Acridine orange, fluorescent stain, scanning electron microscopy, and photography. H. H. A., S. A. S, A.M. E., A. A, M. A. A, D.S. A.. edited the manuscript's final version. All authors have reviewed and approved the final version of the text.

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الكشف الكمي عن غش منتجات اللحوم المصنعة المختلفة ببروتين فول الصويا بالاعتماد على الطرق النسيجية المختلفة

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منتجو اللحوم في مصر ينتجون مجموعة متنوعة من مصنعات اللحوم التي تستخدم بروتينات فول الصويا بسبب ارتفاع أسعار اللحوم. حيث تم اكتشاف مسببات للحساسية في المنتجات التي أعلن أنها خالية منها. ولم تكن نسب فول الصويا في العينات المفحوصة مطابقة للمعايير المصرية لنسب الصويا والتي تبلغ حوالي ١٠%، مما قد يؤثر على صحة المستهلكين. و تم التأكيد على وجود فول الصويا بستخدام طرق الكشف الهستوكيميائى المناعي باستخدام مضاد فول الصويا للأرنب، وتم تحديد نسبة فول الصويا باستخدام الإجراءات النسيجية وتحليل الصور. حيث انه تم الكشف على إجمالي ٥٤٠ عينة بشكل عشوائي من المجمعات الاستهلاكية المختلفة في مدينة سوهاج. وبلغت نسبة فول الصويا في منتجات اللحوم المختلفة، بما في ذلك اللحم المفروم من مستويي الجودة، والنقانق من مستويي الجودة، والكفتة النيئة، وبرجر اللحم البقري من درجتين، وكذلك لحم الدجاج واللانشون، ٨٥%، ٩٠%، ٦٤%. و ٧٦% و ٨٠% و ٦٥% و ٩٧% و ٨٢% و ٦٩% على التوالي. باستخدام الهيماتوكسيلين والأيوسين والأصباغ الكيميائية النسيجية المختلفة، و تم استخدام الفحص المجهري الضوئي للتعرف على الخصائص التركيبية لفول الصويا. و تم تحديد بنية جميع المكونات باستخدام المجهر الفلوري وصبغة أكريدين البرتقالية. حيث تم اكتشاف فول الصويا في عينات اللحوم، وأكدت الأنسجة الوعائية للفلقة وخلايا الاوعية و البرنكماية الغنية بالبكتين هذا الاكتشاف من خلال تقنيات الكيمياء المناعية. و كانت اكثر الطرق نفعا لتقييم لحم البقر المفروم الخام هي المجهر الإلكتروني الماسح.

I. First Section of results: Identification of soybean structures by applying the techniques of electron, scanning, fluorescent and light microscopy methods

The purpose of all the supplemental figures in this section is to make it easier to identify any soy structure in beef products using various histology techniques. Additionally, soya seeds without any mixing with meat products can be described using scanning electron microscopy.

1-Light histological description (general structures of Soybeans have been found in meat samples) by using (light microscopy).

Soybeans have been found in meat samples, and the cotyledons identified them as soybeans. The epidermis and the chalazal endosperm represented the cotyledon's outer layers. The cotyledon's vascular tissue is located between the parenchymal cells (Fig. 1A–G). Protein bodies were abundant in the cotyledon's parenchymal cells, which appeared as acidophilic granules with H&E. (Fig. 1sH). The plaside layer was clearly distinguishable (Fig. 1I, J). An aleurone layer was discovered between the parenchymal cells (Fig. supp1K). The parenchymal cell wall was colored green when stained with Crossomon's trichrome (Fig. 1L).

The cotyledon's parenchymal cells were rich in protein bodies that appeared green with bromophenol blue. The vascular tissue was identified between the parenchymal cells (Fig. 2A). The epidermis covered the cotyledon (Fig. 2B, C). The cotyledon's parenchymal cells contained protein inclusions (Fig. 2D). Pectin-rich parenchymal cells were stained light green by bromophenol blue (Fig. 2D, E). The plaside layer was also recognized using bromophenol blue (Fig. 2F). Parenchymal cells were distinguished by a cell wall, and pectin and vascular tissue was detected between the parenchymal cells (Fig. 2G–I).

The cotyledon's vascular tissue was distinguished from the parenchymal cells that contained pectin (Fig. 3A–D, G, H, I). Starch granules were plentiful in the soybean cotyledon endosperm (Fig. 3E, F).

2-Fluorescent description with acridine orange stain

This part facilitates the research to identify soya structures using these strategies.

Soybean structures were discovered in beef paraffin sections. The palisade layer, mucilage, endosperm, and aleurone were all components of the soybean husk (Fig. 4A–D). Pectin-rich parenchymal cells, vascular tissue, endosperm, and an hourglass layer were all found in the soybean cotyledon (Fig. 4E–L).

3-Scanning histological descriptions (minced soy seed alone, not mixed with other products)

This part facilitates the research to identify soya structures using these strategies. The structure of a ground soybean (Fig. 5) can be analysed on its own to aid in the identification of structures inside meat products. Cotyledon parenchymal cells containing pectin (Fig. 5A, E) and husks (Fig. 4C, D) containing the palisade layer were found in scanned soybean seeds (Fig. 5B). Soybean was found in the scanned meat samples and was characterized by pectin-rich cotyledon parenchymal cells, vascular tissue, and protein bodies (Fig. 6A–G, I–L). Soybean husks were identified in the palisade layer (Fig. 6H).

Figures and Legends

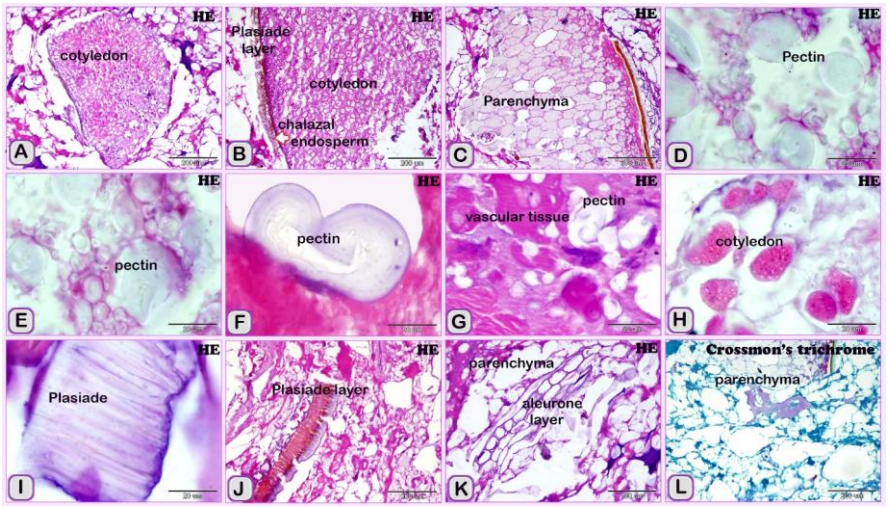

Figure 1: Dentification of Soybean structures were found in the paraffin section of meat samples with H&E stain

A–G: The meat samples contained soybeans, which was recognized by observing cotyledons with parenchymal cells rich in pectin that were stained blue by H&E. The epidermis and chalazal endosperm (arrow) represent the cotyledon's outer layers. The cotyledon's vascular tissue is positioned between the parenchymal cells. H: H&E revealed that the cotyledon's parenchymal cells were rich in protein bodies, which were acidophilic granules. I, J: the palisade layers of the husks were recognized as being from soybeans. K: The alureon of the husk indicated soybeans. L: Crossomon's trichrome stained the parenchymal cell walls green.

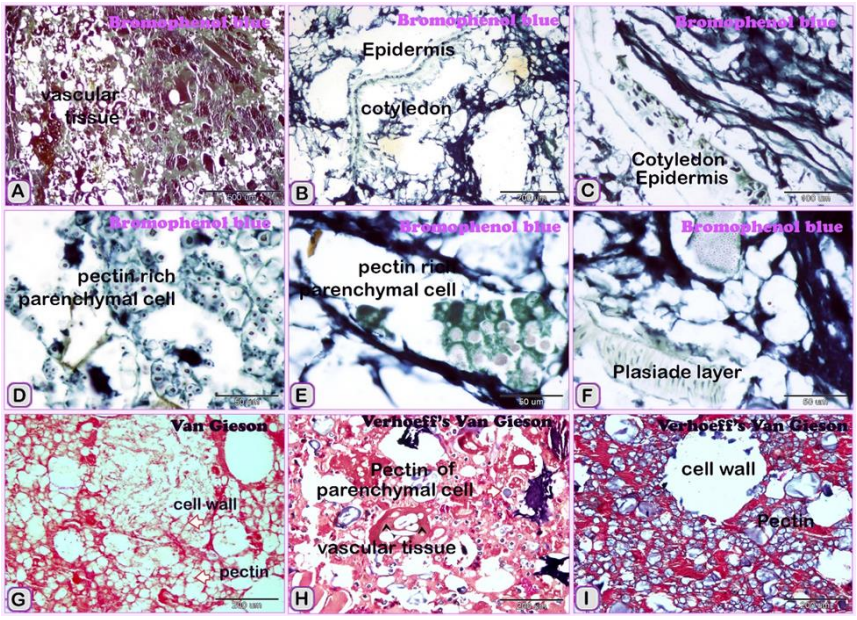

Figure 2: Soybean structures were found in paraffin sections of meat samples that were stained blue by Bromophenol blue(A-F) and Verhoeffs Van Gieson stain (G - I).

A: The cotyledon's parenchymal cells were rich in pectin, which appeared green when stained with bromophenol blue. Note of the cotyledon's vascular tissue. B, C: The cotyledon's epidermis was identified as being from soybeans. D: The pectin-rich parenchymal cells that appeared light green indicated cotyledons of soybeans. E: The parenchymal cells of the cotyledon were rich in protein bodies that appeared green with bromophenol blue staining. F: The palisade layer of the husks, which appeared green with bromophenol blue staining indicated soybeans. G: Cell walls of the cotyledon parenchymal cells appeared red while the pectin appeared pale. H: The vascular tissue (arrowheads) and parenchymal cells (rich in pectin) indicated cotyledons (arrow). I: The cotyledon parenchymal cell's cell wall was red, whereas the pectin was dark blue to black in colour.

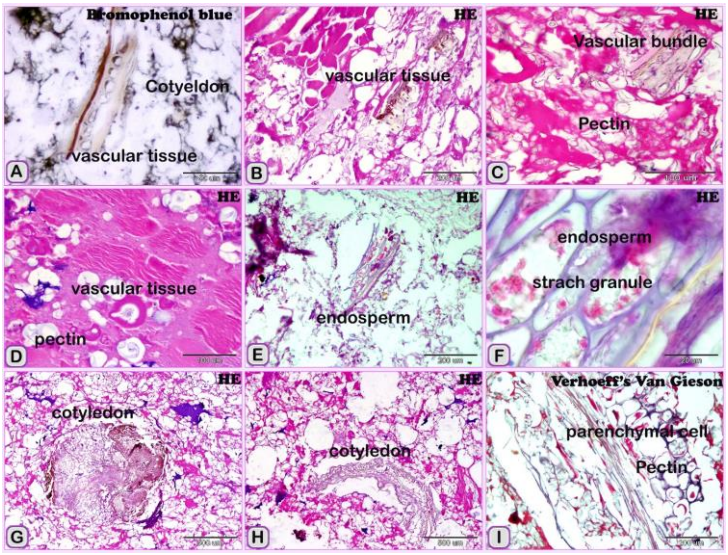

Figure 3: Soybean structures were found in the paraffin sections of meat samples for demonstration of vascular tissue and parenchymal cell with pectin stained with Bromophenol blue (A), Hand E stain (B-H), and Verhoeffs Van Gieson (I).

A–D: Soybean cotyledons were identified according to the vascular tissue and parenchymal cells, which were rich in pectin. E, F: The endosperm of the soybean cotyledon was rich in starch granules. G, H: Soybean cotyledon. I: Cotyledon parenchymal cells rich in pectin

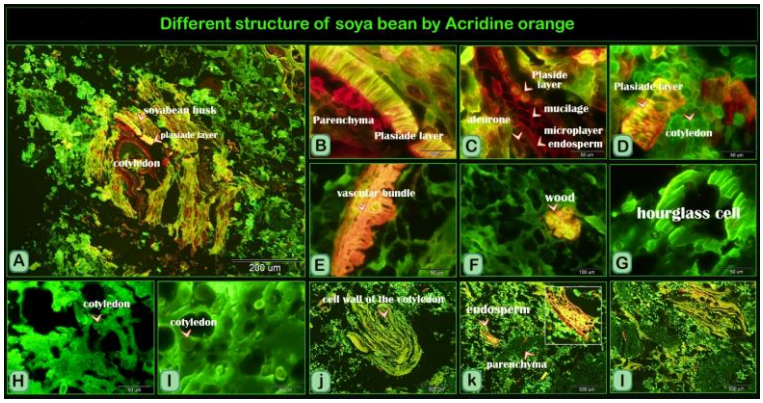

Figure 4: Acridine orange dye was used to stain paraffin-cutting sections, and soybean was recognized.

A–D Soybean husks consisted of a palisade layer, mucilage, endosperm, and aleurone. E–L: Soybean cotyledons contained pectin-rich-parenchymal cells, vascular tissue, endosperm, and an hourglass layer.

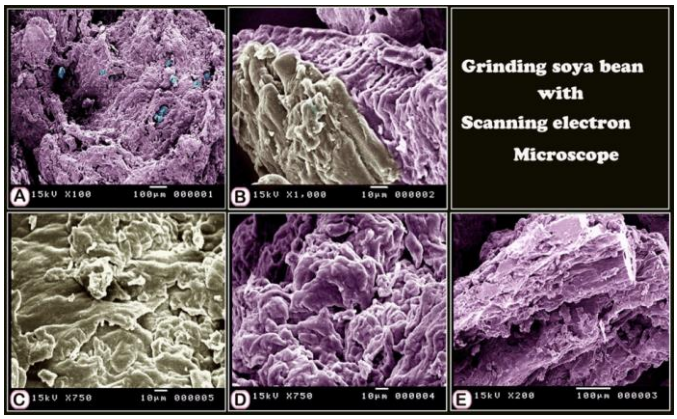

Figure 5: Grinding soybean seed viewed using a scanning electron microscope.

Scanned soybean seed, marked by A, E: cotyledon parenchymal cells (violet) containing pectin (blue). B: the husk's palisade layer. C and D stand for husk.

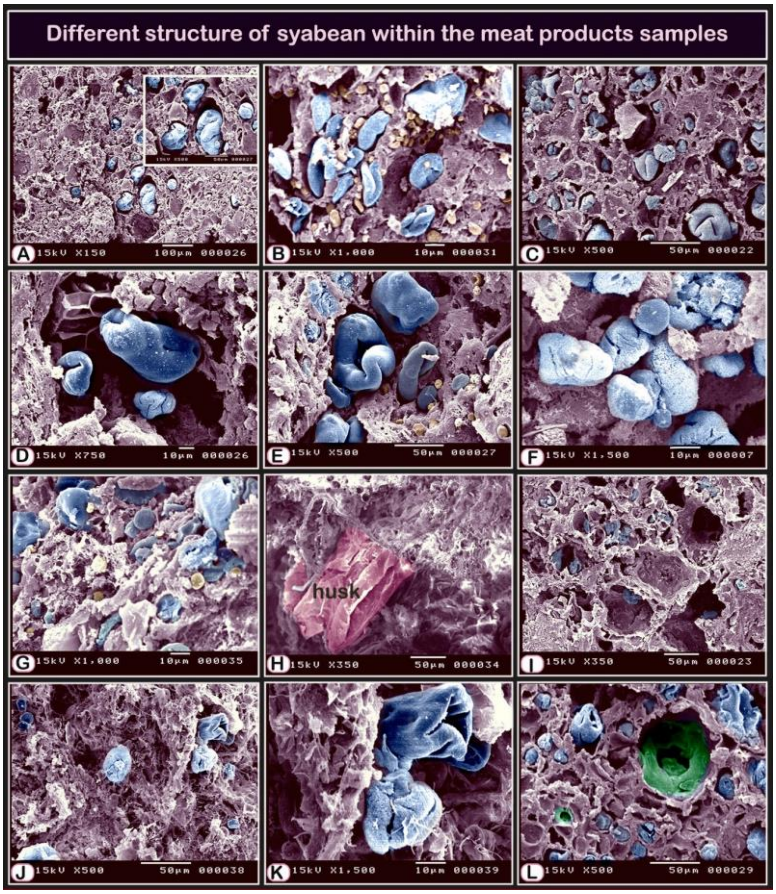

Figure 6: Scanning electron micrographs of soybean found in meat products.
Scanned meat samples showed adulteration by soybeans. A–G, I–L: The cotyledon (violet) was identified with parenchymal cells rich in pectin (blue), vascular tissue (green), and protein bodies (yellow). H: the palisade layer of the husk (pink colour) indicated soybeans.