ORIGINAL ARTICLE

Veterinary Research Forum. 2022; 13 (1) 47 – 53
doi: 10.30466/vrf.2020.115238.2742
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Histological and stereological approaches for detection of tissues and fraud in some meat products

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Article Info

Article history:

Received: 2019 October 02
Accepted: 2020 January 08
Available online: 15 March 2022

Keywords:

Authorized tissues
Histology
Meat product
Stereology
Unauthorized tissues

Abstract

Meat and meat products are highly important sources of protein in the diet. Nowadays, the consumption of meat and meat products has increased owing to modern manufacturing techniques. Due to the economic value of meat, the use of unauthorized tissue is possible in meat products. In some cases, there is fraud in the percentage of meat in meat products to reduce prices. In this study, 34 samples of minced meat, hamburger and sausage were randomly collected from the markets in the northeast of Iran. Then, sections were stained using Hematoxylin and Eosin (H & E), Verhoeff-van-Gieson, Masson's trichrome and periodic acid-Schiff-Alcian blue stains. In this regard, for the first time, the efficacy of stereological technique to determine the percentage of meat listed in sausages and the possible existence of fraud was evaluated. The results showed that, due to the presence of some unusual tissues, histological technique could determine different tissues in meat products. The stereological results of control samples showed a very slight difference; whereas, the results for the samples collected from the city stores showed a distinctive difference regarding the percentage of meat compared to the percentage of label. Skeletal and smooth muscles, blood vessels, nerve, gizzard, adipose tissue, glandular tissue, cartilage, bone, tendon, skin, lymphatic tissues and plant materials were observed. It was confirmed that stereology was a reliable method to determine and confirm the percentage of meat used in meat products.

Introduction

Protein is an essential ingredient in human nutrition that should be produced regularly in the body. Each healthy person needs at least 1.00 g of absorbable protein per kg of body weight per day.[1]

Meat products are called products making up at least half or more than 50.00% of the meat, which may be supplied with additives and preservatives inside natural or synthetic coatings.[2] In addition to minced meat and meat, sausages are also one of the most famous and commonly used meat products used throughout the world, which should be made under health conditions and produced in accordance with standard rules.[3]

Hamburger is also a meat product whose consumption has increased for various reasons including ease of use, presence of meat in its composition and optimal taste. Hamburgers are a significant part of the daily diet of many people at different social levels and age ranges.[4] Today, significant amounts of processed meat products such as sausages and hamburgers are widely used in families.[5]

Food fraud is a major concern in many countries in terms of health risks and economic implications. According to the United States coding instructions, adding any added substance without the consent or knowledge of the customer causing a decrease in the quality, weight, performance or value of the product is called food fraud. Old methods are not enough to detect fraud and laboratory techniques are complex, expensive and time-consuming. In the meantime, the use of tissue sections and the preparation of histological images are widely used in fraud detection.[6]

According to the national standard of Iran, the use of some organs and tissues including lung, spinal cord, glands, visceral cartilages (like cartilages of trachea and lung), fat, skin, head meat, vessels and tendon is

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considered cheating causing them being unusable.[7] Unauthorized animal tissues have low nutritional value proteins and higher microbial load and they can play a role in the transmission of infectious agents such as *Salmonella* and *Escherichia coli*.[8] Owing to the high cost of meat, there is the possibility of fraud in many aspects including the use of unauthorized tissues in minced meat.

Common chemical tests of meat quality control are not capable of detecting fraud in the replacement of unauthorized tissues in meat products; therefore, the use of histological method to identify unauthorized tissues in meat products is recommended.[9,10] The presence of unauthorized tissues in meat products poses a major problem in their identification from the authorized tissues of the product; therefore, the precise method to detect unauthorized tissues in heated meat products is inevitable, since it will pay more attention to the quality of these products.[11]

Furthermore, stereology provides knowledge about shapes, images and stereograms; it has pragmatic methods to recognize images and enables calculation of volumes and volume ratio, the area of samples, the number of particles per unit volume, particle size and unit volume. Thus, this technique is extremely important to obtain reliable quantitative data for various researches in the field of histology. Stereology is generally three-dimensional measurements of microscopic structures. In histological methods, we encounter two-dimensional sections or microscopic images. The science of stereology is used to obtain quantitative information based on the observation and analysis of two-dimensional tissue sections or microscopic images in three-dimensional space. In fact, stereology relies on the knowledge of mathematics and statistical analysis to extract three-dimensional data from two-dimensional images. [12-15]

Since until now no study has been conducted to determine the percentage of meat in food products using the stereological technique, the efficacy of the stereological technique in determination of the sausage meat percentage has been investigated for the first time.

Materials and Methods

Light microscopy. In this study, 20 samples of minced meat, seven samples of hamburger and seven samples of sausage (weigh of each sample was 0.50 kg) were randomly collected from supermarkets. Each sample was divided into three equal parts and then, three pieces from each part were fixed in 10.00% neutral formalin, dehydrated with ethanol series to 100%, cleared in xylene and embedded in paraffin (Merck; Darmstadt, Germany). Blocks (totally 306) were cut into 5.00 µm thickness using a RM2145 microtome (Leica, Wetzlar, Germany). Tissue sections were deparaffinized and stained with Hematoxylin and Eosin (H & E), Masson's trichrome (MT), periodic acid-Schiff-Alcian blue (PAS-AB) and Verhoeff-van-Gieson stains (Merck) for histological observations with a light microscope. The stained sections were sealed with Entellan (Merck). Photos were taken by a CX21 light microscope (Olympus Corp., Tokyo, Japan) equipped with an Olympus camera (U-TVO 63XC; Olympus Corp.).

Stereology. Stereology has never been used to determine the meat percentage in sausage; however, in the present study, it was used for the first time. To perform the stereology and evaluate the efficacy of this technique for the meat percentage determination in food products, sausages were made with 65.00% meat. Then, five samples of these sausages were prepared as control samples. Finally, the stereological technique was performed on control samples as well as five samples of 70.00% sausage meat provided from supermarkets. Stereological studies were conducted by Cavalieri's principle[13,14] to estimate the different areas of various components of sausage, particularly meat. Approximately 10 sections from each specimen (stained with H & E and MT) were selected, several fields from each section were chose (according systematic random sampling) and imaged by the camera attached to the light microscope. More than 50 images were recorded for each sample. Then, a point counting grid with the 0.50 cm distance was used to count points (Fig. 1).

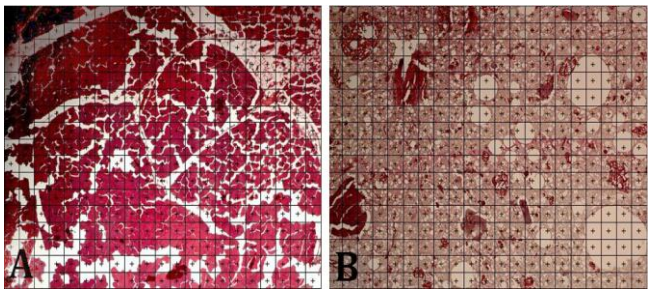

Fig. 1. The grid used to count the tissue components in **A)** standard sausage (control) and **B)** collected sausage samples.

The grid sited on the figures, each area from the various components of sausage was separately counted and an unbiased estimate of the various components of sausage volume was obtained using the following formula:

$$V\,(mm^3) = d \times \sum p \times a(p)$$

where, d is interval between sections or sections thickness (mm), $\sum p$ is total number of points considered on the area of sections and $a(p)$ is the area represented of each point in the grid.

Statistical analysis. The obtained data were analyzed using the SPSS (version 19.0; IBM Corp., Armonk, USA). The statistical analysis was carried out by one-way ANOVA at the level of $p < 0.05$. All results are represented as mean ± standard deviation (SD).

Results

Histological observations. The present study indicated that use of authorized and unauthorized tissues in minced meat, hamburger and sausage is detectable by the histological method. Thus, the histological technique may be a simple and economic tool to evaluate meat adulteration and improve hygiene and meat quality. We used different kinds of staining methods to detect authorized and unauthorized tissues in food products and we concluded that histological methods are a practical technique for routine assessment of authenticity and quality of food products to protect the consumers from fraudulent practice. Light microscopy of minced meat showed various tissue types in addition to skeletal muscle including smooth muscle, gizzard, glandular tissue, skin, adipose tissue, blood vessels and nerve (Figs. 2 and 3). In hamburger, the following tissues were observed: plant tissues, salivary gland, lymphatic tissue (organ), tendon, bone and cartilage (Fig. 4). Also, plant tissues, skin, blood vessels, tendon, adipose tissue and cartilage were observed in sausage (Fig. 5). The presence of some tissues like gizzard, skin, gland and lymphatic organs along with high levels of adipose tissue, bone and tendon confirmed the fraud in these products.

Stereological findings. In this study, the average volume and percentage of the average volume of different components in the standard and collected samples were evaluated. The samples were sausages with different brands. The stereological results of a sample with 65.00% meat showed a very slight difference with percentage on the label of sample. However, the stereological results of the samples collected from the city stores showed a distinctive difference in percentage of the meat compared to the percentage of the label (Tables 1 and 2).

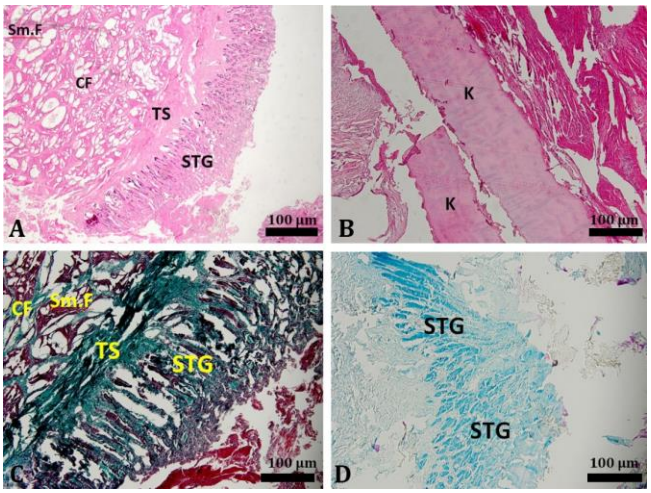

Fig. 2. Various portions of the gizzard have been observed in the minced meat. STG: Straight tubular glands; TS: Tunica submucosa; Sm.F: Smooth muscle fiber; CF: Collagen fiber; K: Koilin. Staining methods: **A)** and **B)** Hematoxylin and Eosin, **C)** Masson's trichrome, and **D)** Periodic acid–Schiff-Alcian blue.

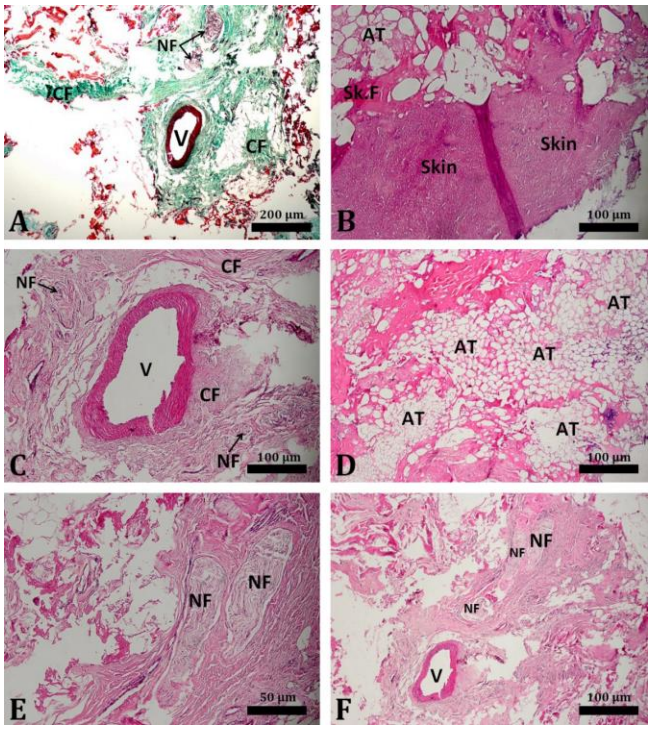

Fig. 3. Blood vessels, nerve, skin and adipose tissue observed in the minced meat. V: Blood vessel; NF: Nerve fiber; CF: Collagen fiber; Sk.F: Skeletal muscle fiber; AT: Adipose tissue. Staining methods: **A)** Masson's trichrome, and **B-F)** Hematoxylin and Eosin.

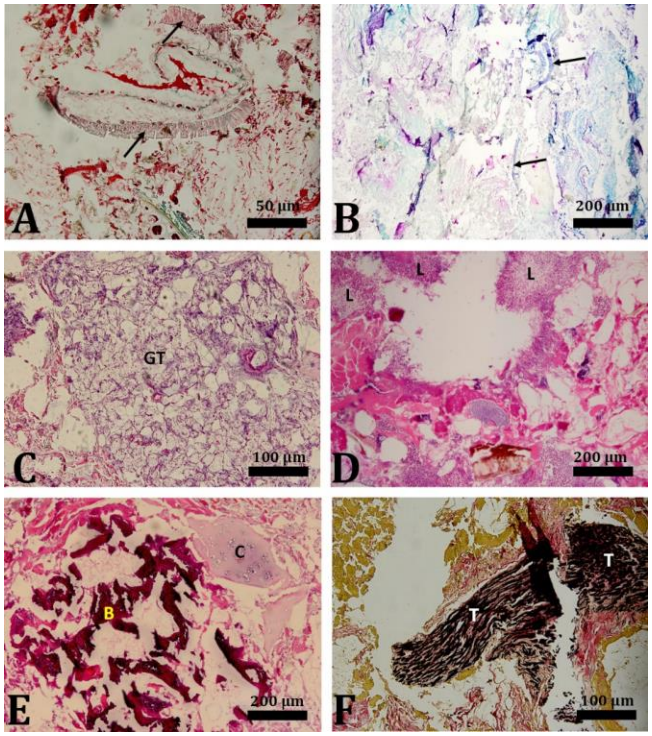

Fig. 4. Unauthorized tissues observed in the hamburger. Arrows: Plant tissues; GT: Glandular tissue; L: Lymphatic tissue or organ; T: Tendon; B: Bone; C: Cartilage. Staining methods: **A)** Masson's trichrome,**B)** Periodic acid–Schiff-Alcian blue; **C, D,** and **E)** Hematoxylin and Eosin, and **F)** Verhoeff-van-Gieson.

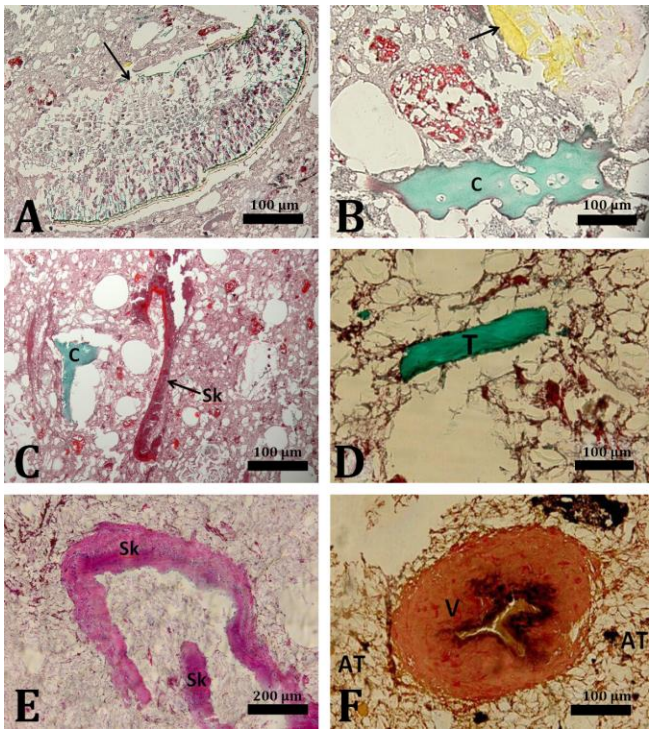

Fig. 5. Unauthorized tissues observed in the sausage. Arrows: Plant tissues; V: Blood vessel; SK: Skin; T: Tendon; C: Cartilage; AT: Adipose tissue. Staining methods: **A, B, C** and **D)** Masson's trichrome, **E)** Hematoxylin and Eosin, and **F)** Verhoeff-van-Gieson.

This discrepancy indicates the existence of fraud in the sausages. Moreover, different tissues including meat, blood vessels, adipose tissue and plant tissues have been observed in sausages.

Discussion

Today, due to lack of meat as one of the major suppliers of protein and micronutrients such as zinc, magnesium and iron to the body, the use of meat products as a protein supplier replacing it with a percentage of meat consumed per capita can meet some of the nutritional needs of the community.

The detection of tissue fraud has been carried out in various meat products such as minced meat, kebab, hamburger, sausage and other products in Iran and other countries and its purpose has been to investigate the presence of additional or unusual tissues and additional tissues associated with plants using the histological technique. The purpose of this study was to complete previous researches and increase the consumer confidence in relation to the accuracy of the label ingredients used in such products.

Histological techniques along with chemical control of meat products are useful and effective to reduce the risk factors threatening the health of consumer. Histological methods have a special advantage over other methods.

Table 1. The mean volume, mm^3 ± SD of the various components of the sausage sample estimated by Cavalier's principle.

Variables	Total volume	Vessels	Adipose tissue	Plant tissues	Meat
Control samples					
Sample 1	101.90 ± 0.36	6.06 ± 0.16	28.38 ± 0.10	3.40 ± 0.09	64.06 ± 0.01
Sample 2	103.80 ± 0.51	6.58 ± 0.17	30.28 ± 0.09	3.82 ± 0.23	63.12 ± 0.02
Sample 3	104.28 ± 0.70	6.14 ± 0.17	30.28 ± 0.14	3.48 ± 0.09	64.10 ± 0.30
Sample 4	104.00 ± 0.39	6.22 ± 0.17	30.04 ± 0.11	3.64 ± 0.09	64.1 ± 0.02
Sample 5	106.08 ± 0.43	7.92 ± 0.21	30.62 ± 0.05	3.44 ± 0.16	64.10 ± 0.01
***p*-value**	0.008	0.012	0.021	0.001	0.031
Collected samples					
Sample 1	99.84 ± 0.36	26.06 ± 0.16	28.38 ± 0.10	39.40 ± 0.09	5.10 ± 0.01
Sample 2	100.44 ± 0.51	26.58 ± 0.17	30.28 ± 0.09	39.82 ± 0.23	4.10 ± 0.02
Sample 3	103.28 ± 0.7	28.26 ± 0.17	30.28 ± 0.14	40.48 ± 0.09	3.10 ± 0.30
Sample 4	104.43 ± 0.39	29.22 ± 0.17	30.47 ± 0.11	40.64 ± 0.09	4.10 ± 0.02
Sample 5	105.08 ± 0.43	29.92 ± 0.21	30.62 ± 0.05	40.44 ± 0.16	4.10 ± 0.01
***p*-value**	0.009	0.015	0.011	0.021	0.001

Table 2. The percentage of volume ± SD of the various components of the sausage sample.

Variables	Meat	Vessels	Adipose tissue	Plant tissues
Control samples				
Sample 1	70.54 ± 0.64	3.66 ± 0.41	22.17 ± 0.44	3.84 ± 0.11
Sample 2	68.08 ± 0.30	4.24 ± 0.34	24.11 ± 0.53	3.74 ± 0.11
Sample 3	68.83 ± 0.14	2.81 ± 0.19	25.71 ± 0.35	3.44 ± 0.11
Sample 4	67.87 ± 0.14	3.36 ± 0.25	25.21 ± 0.23	3.54 ± 0.11
Sample 5	67.87 ± 0.14	3.34 ± 0.37	25.11 ± 0.10	3.56 ± 0.33
Collected samples				
Sample 1	5.54 ± 0.64	20.66 ± 0.20	30.17 ± 0.44	43.84 ± 0.11
Sample 2	6.01 ± 0.30	20.14 ± 0.34	30.11 ± 0.53	43.74 ± 0.11
Sample 3	6.50 ± 0.14	20.01 ± 0.19	32.70 ± 0.35	43.44 ± 0.11
Sample 4	6.50 ± 0.14	20.32 ± 0.25	30.21 ± 0.23	43.30 ± 0.11
Sample 5	6.10 ± 0.14	20.22 ± 0.37	30.11 ± 0.10	43.49 ± 0.33

Histological methods provide a direct diagnosis of a tissue composition in meat products and are used to identify tissue frauds in foods.[16]

The presence of gizzard in minced meat is considered a fraud and confirms the mixing of avian organs in minced meat. The presence of glandular tissue can also be attributed to the use of undesirable meat associated with the head area with salivary glands. This mixing is done to reduce the price of minced meat. Although the presence of adipose tissue is inevitable; but, high fat content without labeling is considered cheating. The unauthorized tissues in hamburger consist mostly of salivary gland, lymphatic tissue (organ), tendon and high amounts of bone and cartilage. The use of unauthorized tissues in hamburger can be considered cheating. The presence of these tissues seems to be due to the use of unfit meat in the production of this product. The unauthorized tissues in the sausage were mainly blood vessels and high levels of adipose tissue, which can be considered fraud.

Sometimes larger vessels and fat are added to the sausage to make it cheaper. This is contrary to the standard regulations. The use of unauthorized animal tissues in meat products is possible due to the economic value of meat itself.

The unauthorized use of animal tissues in meat products including replacing low-price meat species and tissues, vegetable proteins and organic compounds as well as substitution of vegetable fats for animal fats is possible due to the economic value of meat itself.[17]

Various studies have demonstrated the use of unauthorized organs in meat products. In this respect, Sadeghi *et al.* have reported the presence of lung, skin and mammary gland tissues in meat products using histological method.[11] Rokni *et al*., have also showed that salivary gland tissue and nuchal ligament are detectable in Iranian cooked sausage, proving the use of meat obtained from the head of slaughtered animals in meat products.[18] Moreover, Barai *et al*., have compared different approaches to detect frauds in meat products and expressed the efficacy of histological method.[19] Reportedly, Carey *et al.* have found the presence of cardiac muscle, partially defatted tissue as well as soy flour in meat products using histological techniques.[20]

Accordingly, Izadi *et al*., have reported avian skin and adipose tissues in minced meat samples. This study showed that histological technique has the ability to determine the quality and quantity of avian skin mixing values in minced meat products.[21] Further, Sadeghi et al have investigated the quality and health control of meat products using histological techniques.[11]

Correspondingly, Sepehri Eraei has evaluated histological methods to detect adulteration of hamburgers, kebab and minced meat supplied in Tehran. Additive tissues were comprised of avian skin, hyaline cartilage, peritoneal fat and kidney.[22] Prayson *et al*., have also conducted a study into fraud in hamburger using histological techniques and observed blood vessels, skin, bone and cartilage.[23]

Adipose tissue was detectable to a large extent in the studied samples and in all of the stainings, a white color was observed. The excessive use of adipose tissue in meat product samples has been reported formerly.[24]

Larger blood vessels maintain their tissue structures in meat products and are easily recognizable. The present study demonstrated that Verhoeff-van-Gieson staining brushing elastic fiber in black could be a good staining method to diagnose muscular arteries in samples.

Hassanzadeh Yazdi and Sadeghi *et al*., have also reported the presence of vessels in meat products.[11,25]

Plant tissues were observed in different shapes and colors. In the present study, we found that all staining methods could also be used to detect plant tissues. Soybean was found abundantly. Similarly, Jahed Khaniki and Rokni have identified soybean in the sausage and hamburger products.[26]

Due to lack of accuracy in separating meat from bone, in some cases, bone fragments in some samples were observed. Hassanzadeh Yazdi and Abbasy-Fasarani *et al*., have also reported the use of bone in sausage samples.[9,25] Cetin *et al*. found chicken tissue and foreign tissues in ready to sell meat samples and also detected addition of non-meat materials such as ground bone fragment.[27] Other unauthorized tissues found in a number of samples were glands, lymphatic tissues, gizzard and skin. Abbasy-Fasarani *et al*., have reported the presence of mammary gland, lymph nodes, avian skin and gizzard in hamburger samples.[9] Latorre *et al*., have also showed the use of unauthorized tissues in Iranian products including connective tissue, gizzard, adipose tissue, soya, cartilage, ovary, lymph node and gland tissue.[28] The histological examination conducted in the United States on eight different brands of hotdogs showed the presence of connective tissue, blood vessels, peripheral nerve, adipose tissue, plant material, cartilage and bone.[29]

Stereology results showed that it was a reliable method to determine and confirm the percentage of meat used in sausage and the results of study on the standard sample with a 65.00% meat label were desirable. According to the stereology results, it is determined that the volume of meat in the standard sample is between 63.00 and 64.00, which is almost close to the nutritional facts on the label. In non-standard samples, this discrepancy was quite evident and the amount of the meat obtained using the stereological technique in terms of volume and percentage with the label was very different from the reality.

In conclusion, it can be stated that identification of various animal tissues and detection of frauds and mixing of unauthorized tissues and illegal organs in meat products by tissue studies are possible through histological examinations. This study demonstrated that

detection of unauthorized tissues by performing specific stainings including MT, PAS-AB and Verhoeff-van-Gieson, yielded better results. According to the stereological study, it was confirmed that stereology could be a relatively reliable method to detect possible fraud in determining the percentage of meat used in meat products and comparing it to the label.

Based on this study, the presence of unauthorized tissues and incorrect labeling of meat percentage in food products are a serious threat to public health. Therefore, to prevent fraud, more accurate monitoring based on histology and stereology tests should be carried out by health surveillance organizations.

Acknowledgments

This research was supported by the Research Council of the Ferdowsi University of Mashhad, Mashhad, Iran (Grant Number: 3.48461).

Conflict of interest

The authors declare that they have no conflict of interest.

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