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Enhanced Detection and Quantification of Pork Adulteration in Canned Beef Using Real-Time PCR and Thiamine Profiling: A Comparative Study

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IN EGYPT, it is against the law to adulterate meat products, particularly those that are imported and frequently laced with fat or pork. However, it is challenging to identify pork flesh or fat. This study utilized two methods for identifying fat and pork meat in canned beef products. The first approach usedhigh-performance liquid chromatography (HPLC) to estimate the thiamine concentration, and the second applied real-time polymerase chain reaction (RT−PCR) to identify the pork DNA in the meat products. Forty arbitrary samples oflocal or imported canned beef items were collected from a nearby market in Fayoum Governorate, Egypt. Using RT−PCR and thiamine screening methods, the results demonstrated that 50.0% and 25.0% of the samples that were collected were adulterated with pork flesh, respectively. Additionally, RT−PCR was a more efficient approach fordetecting adulteration of canned meat at a level up to > 5.0%,whereas the other methods could not detect adulteration at a level greater than 10.0%.

Keywords: Adulteration, Canned beef, Real-time PCR.

Introduction

Food labeling rules are broken by the adoption of meat species, which is a global issue that affects public health, medical importance, religious and economic significance, and food safety legibility. The term "adulteration" refers to the addition or replacement of meat products with plant proteins, such as soybean, to reduce the amount of animal protein present (Dooley et al., 2004). Moreover, unintended cross-contamination from other meat species utilizing the same equipment or improper, unhygienic human handling are all possible sources of adulteration in meat products (Keyvan et al., 2017; Frank & Hahn, 2016; Abbas et al., 2018). For example, in many countries, meat from other species, such as donkey or dog, meat is considered a type of meat when it is intentionally mixed with cow meat products (Zahran & Hagag, 2015; Yang, et al., 2022). Additionally, pig meat (pork) in Muslim countries, including Egypt, is considered a common type of adulteration of beef meat (Nakyinsige et al. 2012; Dooley et al., 2004; Luo et al., 2009; Li et al., 2022). In an effort to save money, processed beef products can also be adulterated by combining expensive meat with cheaper ones (Ayaz, 2006; Chaudhary & Kumar, 2022). The adulterants used in the majority of the aforementioned types of adulteration of beef byproducts are harder to detect in cooked or ground beef than in fresh or intact meat, and it is easier to identify the source of meat in the beef mixture following grinding, heating, and/or treating operations during manufacturing, such treatment results in significant changes to the texture, color, appearance, and flavor of the meat (Holzhauser and Röder, 2015). As a result, finding

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these adulterants in beef products is a crucial and challenging procedure.

Many studies have recently focused on methods for identifying adulteration in cattle meat products, such as PCR and enzyme-linked immunosorbent assay (ELISA), which identify proteins specific to a particular species. These traditional PCR techniques are simple and useful, but they do not seem to have the sensitivity, quantitative capabilities, or speed of data analysis (Zeitler et al., 2002; Girish et al., 2005; Tanabe et al., 2007;Jia-qin et al., 2008). As a result, for quantitative DNA analysis, RT−PCR is a highly accurate and advised method. Unlike classical PCR, which detects products at the end of the reaction, RT−PCR quantifies DNA using fluorescent emissions generated throughout the reaction during each amplification cycle. Fluorogenic compounds unique to the target amplicon are used in the most effective RT−PCR experiments, and they only produce a fluorescence signal when they bind to it directly or indirectly (Xu et al., 2022).

It was possible to identify pig and chicken derivatives in beef both qualitatively and quantitatively by using a reliable droplet digital PCR method that targets single-copy nuclear genes. By using a constant (transfer coefficient), the mass fraction of the targeted meat was converted to the ratio of DNA copy number (genome/genome) (Yin et al., 2009). A two-tube hexaplex PCR approach was used for actual adulteration, which made it possible to precisely molecularly identify twelve different meat species, including horses, pigeons, cattle, camels, rabbits, cats, turkeys, dogs, chickens, ducks, and geese (Doosti et al., 2011). Multiplex PCR experiments were used to increase the detection limit of the two-tube hexaplex PCR assay. Each PCR contained serial amounts of the DNA mixture of six different species. Genomic DNA extracted from boiled and microwaved beef was used as a template (Hamouda and Abdelrahim, 2022).

Since pigs have a 30 times greater thiamine content than beef, it is common practice to estimate the thiamine concentration of meat and meat products to identify pork. The most accurate technology for estimating the thiamine level of beef is HPLC, although there are other methods that have also been employed (Dawson et al., 1988). Additionally, the goal of this study was to evaluate the efficacy of the RT−PCR approach and HPLC-based thiamine content estimation in identifying adulterated canned beef products.

Materials and Methods

Samples

Part I.

A total of40 canned beef-meat products from eight different brands were purchased from different local markets in Fayoum Governorate, Egypt. The brands of the canned meat products are shown in Table 1.

Part II.

In this part, 40 samples of beef meat products were manufactured experimentally in the Laboratory of Food Science and Technology, Faculty of Agriculture, Fayoum University, using different weights of beef meat and pork meat and ingredients, as shown in Table 2. The samples were divided into 8 treatments, each containing 5 samples, as described in Table 3.

Methods

For sampling, 5 cans were heated at 80 °C for approximately 15 min to melt the fat; the cans were opened, and their contents were wellmixed. The adulteration of the canned beef was determined as follows:

Thiamine content (Vitamin B1).

Thiamine content was determined according to the method described by Dawson et al. (1988).

RT–PCR analysis

Samplepreparation

Beef and pork muscles from a local grocery shop were used to create reference samples. Reference binary mixes containing 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, and 100.0% (w/w) of pork in beef flesh were produced to a final weight of 100 g after the samples were minced separately. Each combination was homogenized using a laboratory knife mill (Grindomix GM200; Retsch, Haan, Germany) utilizing containers and material that had been previously treated with DNA purification buffer after 15 mL was added to sterile phosphate-buffered saline (136 mM NaCl, 1.4 mM KH_2PO_4, 8.09 mM $Na_2HPO_4 \cdot 12H_2O$, and 2.6 mM KCl, pH 7.2). Commercial examples of processed meat products with beef as the primary constituent were homogenized in a Grindomix GM200 laboratory knife mill using material and containers that had first been cleaned with a DNA purification solution. Both the commercial samples and the binary reference were promptly stored at -20 °C until DNA extraction.

TABLE 1. Originand brand of the examined canned beef meat samples

Product brand	Country	Number
Luncheon meat	Jordan	5
Luncheon meat	Brazil	5
Corned beef	China	5
Corned beef	Brazil	5
Corned beef	England	5
Luncheon meat	Jordan	5
Corned beef	China	5
Local Corned beef	Egypt	5

TABLE 2. The ingredients used in the manufacture of the experimental canned meat samples

Ingredients	%
Lean meat	85.67
Fat tissues	11.53
Sodium chloride	1.19
Fresh onion	1.51
Powdered Black pepper	0.01
Laurel leaves	0.09
Total Ingredients	100.00

TABLE 3. The treatments applied in the preparation of experimental canned meat products

Contents		Treatments							
1		2	3	4	5	6	7	8	
%lean meat	**Beef**	100	50	60	70	80	90	95	Zero
	Pork	Zero	50	40	30	20	10	5	100
% Fat	**Beef**	100	50	60	70	80	90	95	Zero
	Pork	Zero	50	40	30	20	10	5	100

DNA extraction

The Wizard technique (Cai et al., 2022) was used to extract DNA with a few modifications. Briefly, a sterile reaction tube measuring two mL was filled with 100 mg of eachground and homogenized sample, 860 mL of the extraction buffer, 100 mL of 5 M guanidine hydrochloride solution, and 40 mL of proteinase K (20 mg/mL). The suspension was centrifuged (15 min, 18,514 g),and 500 µL of the supernatant was combined with 1 mL of Wizard DNA purification resin after being incubated at 60 °C for three hours with periodic stirring. The mixture was eluted using a column, and the resin was then rinsed with 2 mL of 80.0% (v/v) isopropanol solution. After the column was dry, the DNA was extracted by centrifugation using 100 µL of Tris-EDTA buffer at 70 °C and transferred to a fresh reaction tube. The extractions were performed in duplicate for each binary mixture. The quality of the extracted DNA was then tested by electrophoresis in a 1.0% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) for 40 min at 120 V, staining with ethidium bromide (0.4 g/mL) for 5 min, and destaining in distilled water for 20 min.

DNA quantification and purity

Spectrophotometry was used to quantify the DNA using a Shimadzu UV-1800 spectrophotometer. The DNA concentration was determined by measuring the absorbance at 260 nm (one absorbance unit = 50 ng/L of dsDNA). When the purity of the extracts was measured using the absorbance ratio at 260 and 280 nm, values ranging from 1.7 to 2.0 were identified.

Oligonucleotide primers

The oligonucleotide sequences of primers used in this work are listed in Table **IV**. The primers used were synthesized by Macrogen.

Real-time PCR

RT□PCR amplification was performed in 20 µL of DNA extract, 1 iQTMSYBR® Green Supermix, and 500 nM of each primer (Table 4), which were generated in parallel reactions for each target sequence. RT–PCR was performed on a fluorometric thermal cycler (iCycler iQ™ Real-time) detection system under the following conditions: 95 °C for 5 min; 45 cycles at 95 °C for 30 sec and 65 °C for 1 min; and collection of fluorescence signals at the end of each cycle. For the melting curve data, the temperature was increased by 0.5 °C from 65 °C to 94 °C. The data were collected and processed using iCycler iQ™ Real-Time Detection System Software version

Results

As shown in Fig.1, the thiamine contents in the pork and beef meat were 82.84 and 2.49 mg/100 g of total nitrogen(TN)in terms of dry weight, respectively. Additionally, the data shown in Fig. 2 emphasize thatthe lowest thiamine content was found in the samples from treatment 1 (0.91 mg/100 g of TN), which had 100.0%beef and zero pork, while the highest value was observed in the samples from treatment 8 (22.01 mg/100 g of T N), which had zero beef and 100.0% pork. Moreover, the thiamine contents in the samples from treatments 2, 3, 4, 5, 6, and 7 were 9.65, 8.42, 5.4, 4.34, 3.0 and 2.75 mg/100 g of T N, respectively.From the results in Fig. 3, it was clear that the T N percentages of thiamine were low in samplesA, B, C, D, F and H at 1.85, 1.79, 2.05, 2.1, 2.15 and 2 mg/100 g of T N, respectively. However, in samples E and G, the thiamine contents were 4.01 and 4.03 mg/100 g of T N, respectively, which are higher than the suggested values for pork detection.

RT–PCR based on SYBR Green dye was used to quantitatively analyze the quality of the treated pork. Binary meat mixes, including known proportions of pork and beef-meat, were utilized to create an adjustment modelwith strong PCR efficiency and linear correlation that was normalized to values ranging from 5.0 to 100.0% (Fig. 4 A and 4B). Fig. 4 (A and B) and Fig. 5 (A and B) show that a calibration curve based on real-time PCR normalization is necessary for appropriate quantitative analysis. Considering that processed meatproducts generally have several ingredients, including those from vegetable sources, and that different processing treatments might affect target gene amplification, the use of an endogenous control enables these variations to be controlled.

Figure 6 shows that RT–PCR was more sensitive and precise than normal PCR since RT–PCR with SYBR Green I enabled the identification and quantification of pork DNA in the meat blends. We were able to ascertain that four of the eight analyzed samples were made using hog fat or flesh by using the SYBR Green I method for real-time PCR.In regard to the estimation of the addition of pork to commercial processed meat products,the technique was further used to quantify the eight commercial canned-meat items after being designed and verified (Table 5 and Fig. 7). The RT–PCR results showed that the proportions of pork-meat or fat in the samples that

TABLE 4. Sequences of Primers used for RT–PCR amplification

Specific gene	Primers	Sequence 5′–3′	References
Cytb	Pork-F	ATGAAACATTGGAGTAGTCCTACTATTTACC	Dooley et al. (2004)
	Pork-R	CTACGAGGTCTGTTCCGATATAAGG	
	Beef- F	GCCATATACTCTCCTTGGTGACA	Krčmář and Renčová (2001)
	Beef- R	GTAGGCTTGGGAATAGTACGA	

TABLE 5. Results for the validation of RT–PCR in the evaluation of the imported and local canned beef samples (A–H) and the experimental samples (1—8)

Imported and Local canned beef s samples	* ct beef	** ct pork	*** Δ ct	**** log con.	% pork
A	14.63	0	-14.63	0	0
B	17.99	0	-17.99	0	0
C	19.29	26.45	7.15	0.68	4.84
D	19.42	26.25	6.83	0.79	6.23
E	20.49	26.29	5.80	1.14	13.74
F	14.49	0	-14.49	0	0
G	19.48	25.04	5.56	1.22	16.45
H	14.69	0	-14.69	0	0
Experimental Samples (Treatments 1 – 8).					
1	21.72	.	0	0	0
2	21.40	26.32	4.92	1.69	50
3	21.01	25.49	4.48	1.60	40
4	19.50	24.02	4.52	1.48	30
5	21.82	24.37	2.56	1.30	20
6	21.21	26.34	5.14	1	10
7	19.38	26.56	7.19	0.69	5
8	0	22.66	22.66	2	100

*** ct beef = cycle threshold of beef,**ct pork =cycle threshold of pork,***Δ ct = ct pork - ct beef,****log con. of pork = logarithm concentration of pork**

were discovered to contain pork meat or fat were 4.84, 6.22, 13.74 and 16.45%, respectively. Only A, B, F, and H were positive for pork amplification by RT−PCR in the samples with no stated pig content, indicating that they were in accordance with the labeling results.

Discussion

The current observation (Fig. 1) indicated that the thiamine content of the pork was more than 30 times that of the beef. These findings coincided with those of Poel et al. (2009) and Lombardi-Boccia et al. (2005), who reported that the thiamine contents in pork and beefwere 0.9 and 0.02 mg/100 g (fresh weight), respectively. Fig. 2 shows that the thiamine content was directly proportional to the content of pork and inversely proportional to the beef content in the samples. The thiamine contents in the fresh beef meat and the samples from treatment 1 were 2.49 and 0.91 mg/100 g of T N, respectively (Fig. 1). The reduction in thiamine content in the samples of treatment 1, which were manufactured with 100.0% beef/zero content pork, could be attributed to the process of heating during laboratory manufacturing, whichreduced the thiamine content by 74.7 and 83.2% in the canned-beef and canned pork, respectively. This was comparable to that achieved byLombardi-Boccia et al. (2005), who attributed the loss of thiamine content to heat processing, and the losses differed according to the type of heat treatment. Furthermore, the losses were approximately 15.0-40.0% by boiling, 40.0-50.0% by frying, 30.0-60.0% by roasting, and 50.0-70.0% by canning. Notably, the decrease in thiamine content due to the canning process was greater in beef than in pork, which could be attributed to the high fat content in pork, which may isolate thiamine from oxygen and protect it from damage during canning. As mentioned above, the thiamine content in sample treatment 7, which contained 5.0% pork, was 2.87 mg/100 g of T.N., while that in pure canned beef (treatment 1, which contained zero pork) was 0.91 mg/100 g of T.N., and that in fresh beef contained 2.49 mg/100 g of T.N. It could be assumed that 2.5% thiamin was a sufficientindicationof pork adulteration in canned meat; in other words, this level could be used as a rough cutoff for pork adulteration in canned meat.

The results in Fig. 3 show that the thiamine content was lower than the suggested level of 2.50 mg% 100 g T.N. for the detection of the presence of pork. Additionally, for samples E and G,the thiamine contents were 4.01 and 4.03 mg/100 g of T N, respectively, which are higher than the suggested values for pork detection, indicating that samples 5 and 7 E and G (imported canned beef) may contain high amounts of pork that is adulterated with pork muscle tissues.Fig. 4 (A and B) and Fig. 5 (A and B) show that a calibration curve based on real-time PCR normalization is necessary for appropriate quantitative analysis. Considering that processed meatproducts generally have several ingredients, including those from vegetable sources, and that different processing treatments might affect target gene amplification, the use of an endogenous control enables these variations to be controlled. For this purpose, the application of the ΔΔCt method to construct a calibration model was proposed by calculating $\mathbf{\Delta\Delta Ct = Ct_{pork} - Ct_{beef}}$ (where Ct_{beef} and Ct_{pork} are the cycle thresholds for beef and pork systems, respectively, obtained through the amplification of binary model mixtures) (Fig. ٤A and 4B). A calibration curve can be obtained by charting the Ct vs. the pork-meat logarithm %. (Fig. 6). With this method, additional pork meat can be estimated at concentrations ranging from 5% to 100%, which has an active range that is linear at least 3 orders of magnitude (Pathare and Roskilly 2016), standards for real-time PCR studies, and a powerful correlation coefficient (R_2=0.9893).

The observations in Fig. 6 indicate that hog flesh or fat was present in almost 50.0% of the studied samples (Table 5). In contrast, the thiamine vitamin evaluation test revealed that only two (the E and G samples) of the eight samples under examination appeared to contain hog flesh. According to our findings, the thiamine vitamin method is less sensitive and specific than PCR methods for detecting adulterated pork meat in meat mixtures. This is because PCR methods rely on the detection of pork DNA, whereas the thiamine assessment method solely evaluates the thiamine content. This result was in line with the assumptions made by Chen et al. (2020) and Wibowo et al. (2023), who postulated that PCR techniques, in particular, RT−PCR, are highly specific and effective in identifying the adulteration of beef with other meat species (Hassanin et al., 2018; Chen et al., 2020; Galal-Khallaf, 2021).

The technique was further used to quantify eight commercial canned-meat items after they were designed and verified. Table 5 and Fig. 7 show the RT−PCR results. The samples that were

discovered to contain pork meat or fat were C, D, E and G, and the proportions of porkmeat or fat were 4.84, 6.22, 13.74 and 16.45%, respectively. Only A, B, F, and H were positive for pork amplification by RT−PCR in the samples with no stated pig content, indicating that they were in accordance with the labeling results.Because RT−PCR with SYBR Green I allows for the detection and quantification of pork DNA in meat mixes, RT−PCR is much more sensitive and precise than standard PCR (Hassanin et al., 2018; Chen et al., 2020; Galal-Khallaf, 2021). Through the use of the SYBR Green I technique in real-time PCR, we were able to determine that four out of the eight tested samples were produced from hog fat or flesh. This means that approximately fifty percent of the tested samples contained pork flesh or fat (Table 1). Conversely, out of the eight examined samples, only two (E & G samples) seemed to contain pork meat when the thiamine vitamin evaluation test was used. According to our findings, the thiamine vitamin method is less sensitive and specific than PCR methods for detecting adulterated pork meat in meat mixtures. This is because PCR methods rely on the detection of pork DNA, whereas the thiamine assessment method solely evaluates the thiamine content. This result was in line with the assumptions made by Chen et al. (2020) and Wibowo et al. (2023), who postulated that PCR techniques, in particular, RT□PCR, are highly specific and effective in identifying the adulteration of beef with other meat species. Additionally, RT−PCR with SYBR Green I allow for the detection and quantification of pork DNA in meat mixes, and RT−PCR is much more sensitive and precise than standard PCR (Hassanin, et al., 2018; Chen, et al., 2020; Galal-Khallaf, 2021). Through the use of the SYBR Green I technique in RT−PCR, we were able to determine that four out of the eight tested samples were produced from hog fat or flesh. Approximately fifty percent of the tested samples contained pork flesh or fat (Table 1). Conversely, out of the eight examined samples, only two (E & G samples) seemed to contain pork meat when the thiamine vitamin evaluation test was used.

Conclusion

This study successfully demonstrated two methods—high-performance liquid chromatography (HPLC) and real-time polymerase chain reaction (RT−PCR)—for detecting adulteration in canned beef products, particularly identifying the presence of pork fat or flesh. The analysis of 40 randomly selected samples from Fayoum Governorate revealed significant adulteration, with 50% of the samples containing pork meat, as confirmed by RT−PCR, and 25% detected through thiamine concentration screening. Notably, RT−PCR was found to be a more reliable and sensitive technique for identifying pork adulteration, capable of detecting contamination at levels as low as 5%. This highlights the importance of employing advanced methods like RT−PCR to ensure food authenticity and protect consumers from fraudulent practices in meat products.

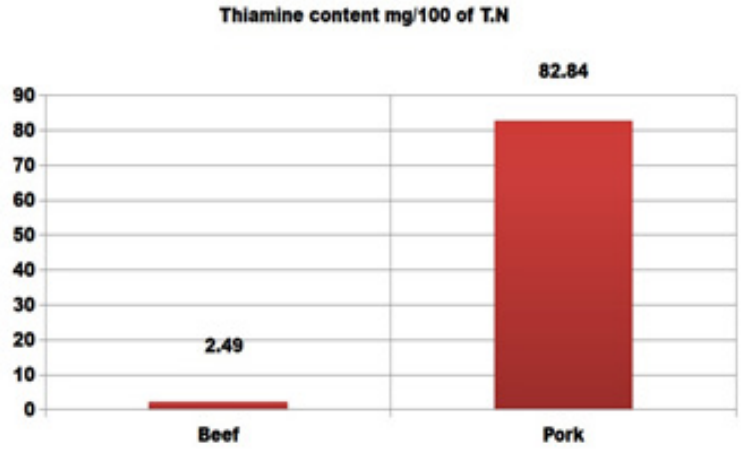

Fig. 1: Thiamine content (mg /100 g of T.N) in the fresh beef and pork

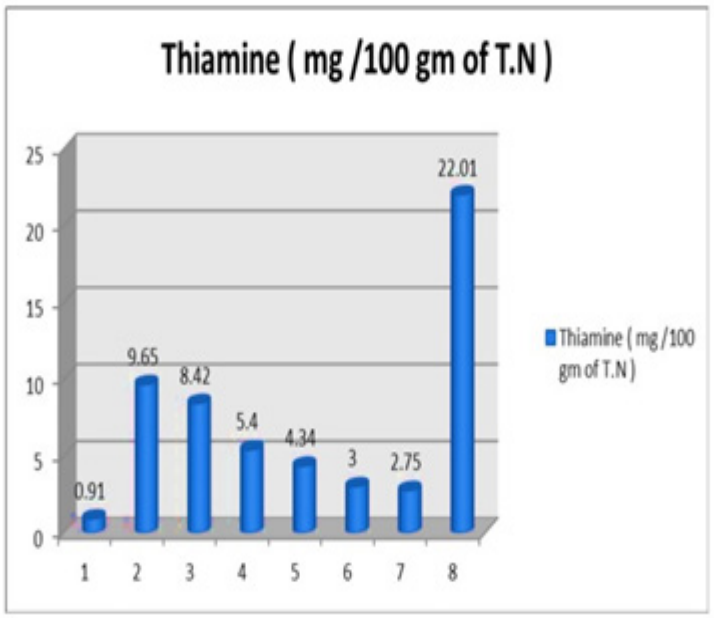

Fig. 2: Thiamine Content (mg/100 g/T.N) of laboratory Canned Meat Products with Different Levels of Beef and Pork (Treatments 1 – 8) on dry weight basis

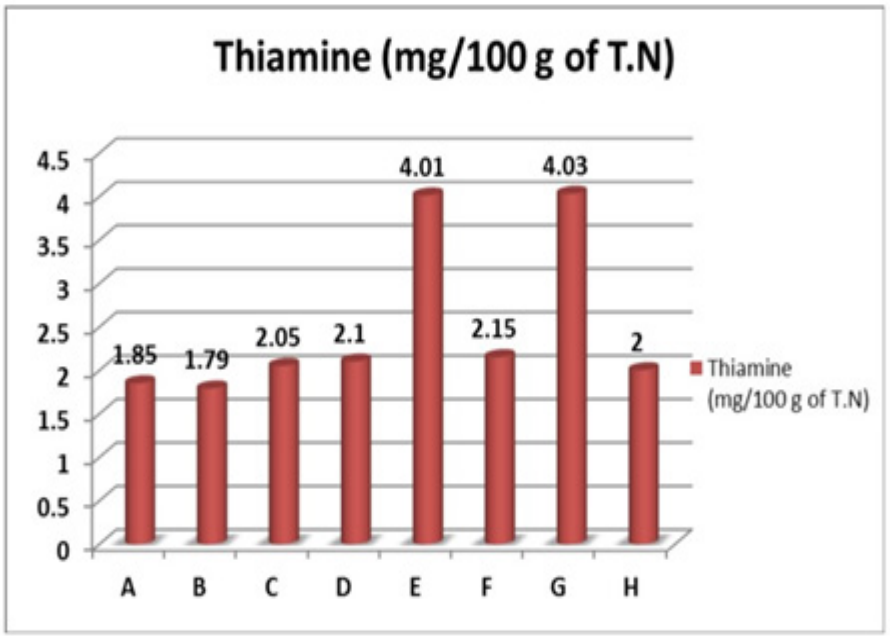

Fig. 3: Thiamine content (mg /100 g of T.N) of some imported and local canned meat products

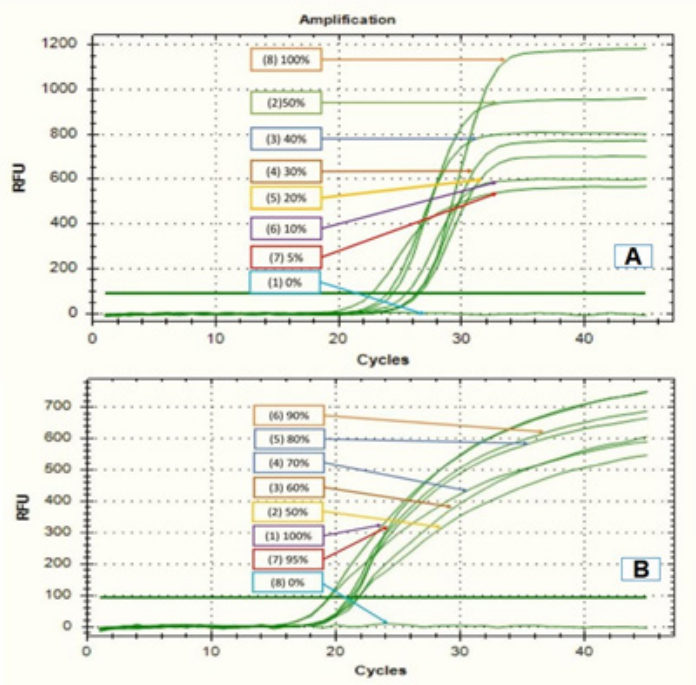

Fig. 4. Curves of amplification for binary reference mixtures (5 to 100% of canned pork meat treatments 1to 8 pork (A) canned beef meat (B)) using RT–PCR with SYBR Green I dye

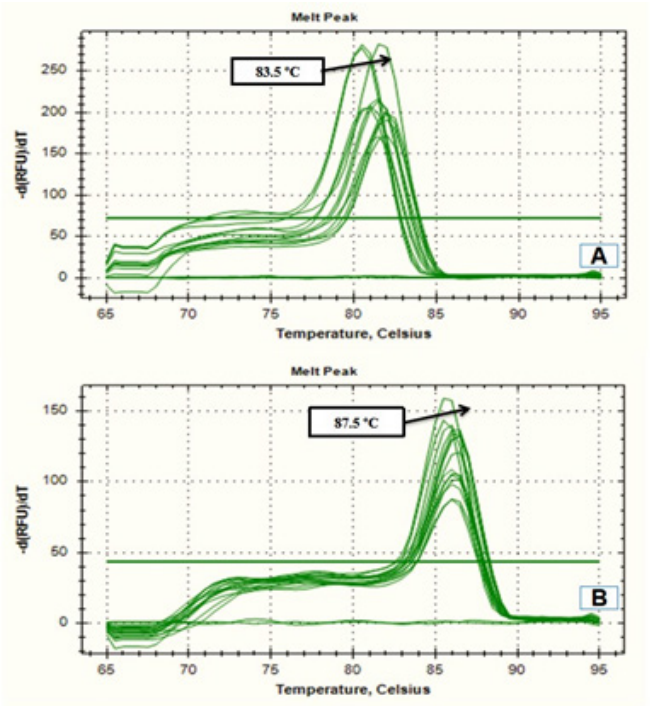

Fig. 5. RT–PCR melting curves for binary reference mixes (5 to 100%) of canned pork (A)and canned beef (B) using SYBR Green I'm a pork dyer

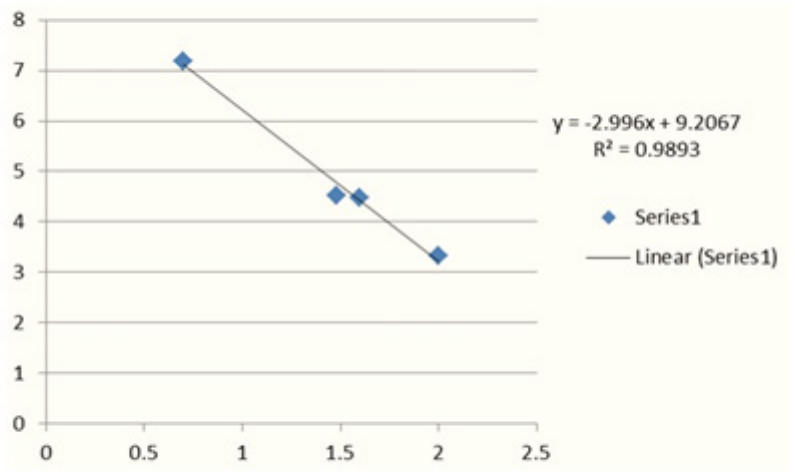

Fig. 6. Using pork-meat amplification as a reference gene and the ΔΔCt technique, a normalized calibration curve was created for the determination of the degree of adulteration in beef meat

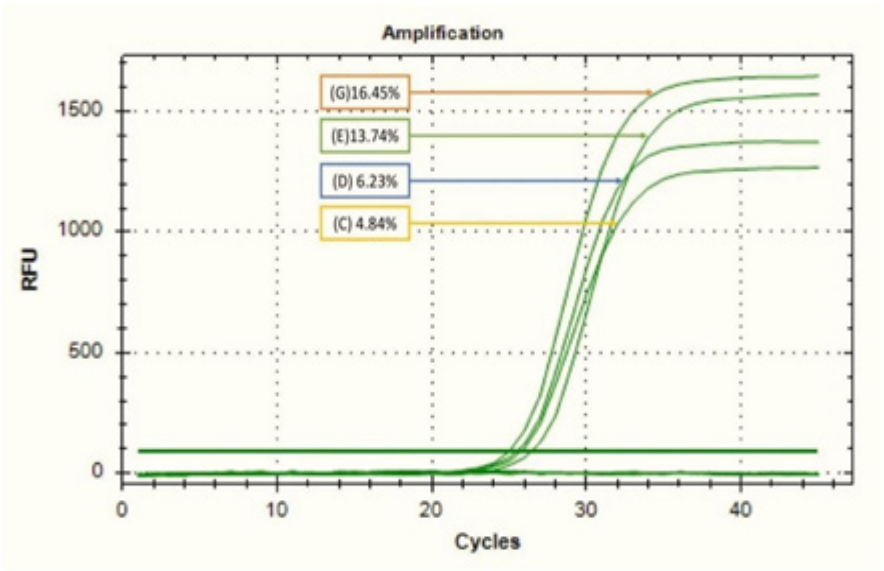

Fig. 7. Amplification curves of imported and local canned meat products (A to H) by RT–PCR with SYBR Green I dye for pork

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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تحسين الكشف والقياس الكمي لغش لحم الخنزير في لحوم البقر المعلبة باستخدام تفاعل البوليميراز المتسلسل في الوقت الحقيقي وملف الثيامين: دراسة مقارنة

في مصر، يعد غش منتجات اللحوم مخالفًا للقانون، وخاصة تلك المستوردة والتي غالبًا ما تكون مخلوطةً بالدهون أو لحم الخنزير. ومع ذلك، من الصعب تحديد لحم الخنزير أو دهنه. استخدمت هذه الدراسة طريقتين لتحديد دهون ولحوم الخنزير في منتجات لحوم البقر المعلبة. استخدمت الطريقة الأولى كروماتوغرافيا السائل عالية الأداء (HPLC) لتقدير تركيز الثيامين، وطبقت الطريقة الثانية تفاعل البوليميراز المتسلسل في الوقت الحقيقي (RT–PCR) لتحديد الحمض النووي للحم الخنزير في منتجات اللحوم. تم جمع أربعين عينة عشوائية من لحوم البقر المعلبة المحلية أو المستوردة من سوق قريب في محافظة الفيوم، مصر. باستخدام طرق RT–PCR وتقدير الثيامين، أظهرت النتائج أن ٥٠,٠٪ و ٢٥,٠٪ من العينات التي تم جمعها كانت مغشوشة بلحوم الخنزير، على التوالي. بالإضافة إلى ذلك، كان RT–PCR هو النهج الأكثر كفاءة للكشف عن الغش في اللحوم المعلبة بمستوى يصل إلى > ٥,٠٪، في حين أن الطرق الأخرى لم تتمكن من الكشف عن الغش بمستوى أعلى من ١٠,٠٪.