Journal of Scientific Research in Medical and Biological Sciences
ISSN 2709-0159(print) and ISSN 2709-1511 (online)
Volume 3, Issue 1 Article 3
DOI: https://doi.org/10.47631/jsrmbs.v3i1.441

DEVELOPMENT AND EVALUATION OF ELISA KITS BASED ON PROTEOMICS TECHNOLOGY FOR DETECTION AND SEMI-QUANTIFICATION OF MEAT SPECIES ADULTERATION

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ARTICLE INFO

Received: 23 January 2022
Revised: 12 March 2022
Accepted: 12 March 2022

Keywords:
DNA-Based Detection, Proteomics Technology, Meat Species Adulteration.

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ABSTRACT

Purpose: *This study aimed to develop ELISA kits for the detection of meat species adulteration in raw and processed meat and evaluate its specificity and sensitivity.*

Method: *We identify the unique peptide markers based on the proteomics approach. Subsequently, we prepared specific antisera for beef, pork, horse, sheep, chicken and developed ELISA kits.*

Results: *Evaluation of the developed kits in testing crooked reference sample mixtures revealed that the developed ELISA kits showed 100% specificity with no cross-reactivity detected for the non-target species. In terms of sensitivity, the developed ELISA kits were able to consistently detect pork in the sample mixtures and horses at levels down to 5% w/w. Although sheep and chicken were detected at levels as low as 1% w/w. The beef-specific ELISA test showed greater sensitivity.*

Conclusions: *It is concluded that the developed ELISA kits are a promising tool but further studies are still required for validation of the developed kits and comparing its results with the commercially used kits before entry into the production phase.*

INTRODUCTION

Food labeling legislation demands detailed and reliable information concerning the content of any food product; this information is likely to influence consumer choice based on moral, religious, ethical, dietary, or health factors. Enforcement of food labeling regulations requires robust and validated analytical methods to verify the authenticity of meat-based foods. At present, meat authentication is largely reliant on DNA-based detection technologies (O'Mahony, 2013). PCR methods offer great specificity and sensitivity, even in some heat-treated foods and feed. However, both nuclear and mitochondrial DNA are sensitive to heavy processing methods (Sakalar et al., 2012) and therefore DNA-based technologies cannot always be robustly applied to highly processed foods, especially when quantification is necessary. ELISA methods are also used for meat speciation (Chen & Hsieh, 2000), but there are also a number of limitations with ELISA-based testing such as poor specificity, matrix interference, or the effect of different food processing procedures on antigen availability.

Proteins are substantially more diverse in terms of their physicochemical properties when compared with nucleic acids and some will survive the heating and other conditions associated with highly processed foods. Therefore, many proteins will suffer only partial degradation under such treatments, resulting in large fragments still present after processing. The primary structures (amino acid sequences) of these protein fragments can be analyzed using proteomics tools to determine the species of origin. Proteomics workflows have been developed in recent years using high-resolution MS to determine the species of origin of animal-derived ingredients in food and feed products (Montowska et al., 2014). This study aimed to use the proteomics approach to identify the unique peptide markers for five meat species to prepare specific and sensitive antisera against each to be used in developing ELISA kits to detect meat species adulteration and evaluate the developed kits in testing cooked reference sample mixtures.

METHODOLOGY

Sample collection

Different meat species standard samples of known origin (beef, pork, horse, sheep and chicken) were supplied by the General Organization of Veterinary Services, Egypt. Other meat species commercial samples (raw meat, cooked meat, and thermally processed) were obtained from different butchers and supermarkets. All samples were stored at -20°C until used.

Preparation of meat species standard samples:

Preparation of thermally processed meat laboratory:

The different meat species were laboratory prepared to induce the industrial processing effects. Meat samples were minced firstly. Homogenized meat was cured in NaCl salt (20% w:w) for 4 days at 4°C. Following this curation, sufficient water was added to cover the meat samples and the samples were simmered for 20 min on a hot plate with stirring using a magnetic bar. The laboratory-cooked meat samples were then autoclaved with high temperature and pressure at 121°C for 15 min. These processed meat samples were stored at -20°C for further use.

Preparation of meat species mixture:

For the determination of LOD, different meat species mixtures were prepared by weighting respective amounts (10 mg) of meat to obtain beef samples spiked with various weight ratios of other meat species (10%, 5%, 1% and 0.5% w/w) in a 2 mL Eppendorf tube and then laboratory-processed (table 1).

Table (1): meat species mixture for determination of LOD.

	Beef	Pork	Horse	Sheep	Chicken
Sample 1	100%				
Sample 2		100%			
Sample 3			100%		
Sample 4				100%	
Sample 5					100%
Sample 6	90%	10%			
Sample 7	90%		10%		
Sample 8	90%			10%	
Sample 9	90%				10%
Sample 10	95%	5%			

Sample 11	95%		5%		
Sample 12	95%			5%	
Sample 13	95%				5%
Sample 14	99%	1%			
Sample 15	99%		1%		
Sample 16				1%	
Sample 17					1%
Sample 18	99.5%	0.5%			
Sample 19	99.5%		0.5%		
Sample 20	99.5%			0.5%	
Sample 21	99.5%				0.5%

Method of meat proteins extraction from both standard and commercial samples:

For each 1 gram of meat species sample, 10 ml of 50 mM Tris-HCl buffer, pH 8.0 were added then the sample was homogenized by using a Vortex for 2 minutes. The homogenate sample was centrifuged at 10,000 g for 20 minutes at 4 C. The supernatant that constituting the sarcoplasmic extract was discarded in which most of the sarcoplasmic proteins become denatured and insoluble with thermal processing. most of the sarcoplasmic proteins. The collected pellet was suspended in 10 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M urea and 1 M thiourea, and then homogenized in a Vortex for 5 minutes for extracting the myofibrillar proteins. Most myofibrillar proteins remained in the extract in both fresh and thermally processed meats. The homogenate was centrifuged at 10,000 g for 10 minutes again. The supernatant containing the myofibrillar extract was collected and filtered through 0.45 m membrane filters (Millipore) before use. The protein concentration of the final extract was determined using the Bradford method.

Development of anti-species antibodies:

Construction of a database of species-specific peptides:

All database searches were performed based on our selection criteria as the following;

1. Myofibrillar proteins: Myofibrillar proteins constitute a more reliable and robust source of peptide biomarkers because most of the sarcoplasmic proteins undergo denaturation after heat treatment making them insoluble. In contrast, most myofibrillar proteins remained in the same extract in both fresh and cooked meats, giving rise to the generation of the same peptide biomarkers in all cases.
2. Well distributed in muscles and other organs: myosin
3. Survive industrial processing to identify proteins present in heavily processed food products.
4. Solubility in salt:
5. Long amino acid chains: nearly 18-20 amino acids

Using bioinformatics to compare amino acid sequences across a range of relevant meat animal species to find unique marker peptides: We created a database of unique peptides for beef, pork, horse, sheep, chicken, and rabbit using the UniProt database (www.uniprot.org). The full amino acid sequences of the proteins identified from each species were compared across all vertebrates to select a list of species-specific peptides for beef, pork, horse, sheep, chicken.

Initially, we searched against a single fasta file containing the UniProt reviewed sequences for species proteins to produce a conclusive list of identified abundant proteins. The amino acid

sequences of selected proteins that were most confidently identified across the six species were then obtained for all species of interest, namely cow (Bos taurus), pig (Sus scrofa), horse (Equus caballus), sheep (Ovis aries), chicken (Gallus gallus), and rabbit (Oryctolagus cuniculus), from either UniProt or through BLAST (the Basic Local Alignment Search Tool) searching the NCBI non-redundant (NCBInr) database. This list of protein sequences was then used to construct a database containing only the unique tryptic peptides from each protein across the species. Firstly a peptide-picking algorithm was used to compare the in silico tryptic peptides of each protein across the different species entries. These unique tryptic peptides were then searched using

BLAST to ensure that they were truly unique between all isoforms of all proteins currently in the databases for the six species (cow taxid:9913, pig taxid:9823, horse taxid:9796, sheep taxid:9940, rabbit taxid:9986, chicken taxid:9031).

Synthesis of multiple antigen peptides (MAP) to be used as an immunogen:

The immunogens used were synthetic unique marker peptides of the five species. They were constructed in multiple antigen peptide (MAP), tetra branched by: Hangzhou Dangang Biological Technology Co., Ltd., China.

Performing immunization protocol to develop anti-species antibodies:

This protocol is optimized to produce antiserum in the shortest possible time. The immunization schedule was performed using three NZW SPF rabbits (New Zealand White rabbits that are Specific Pathogen-Free) for each immunogen. Rabbits were injected subcutaneously (SC) with the immunogen as an emulsion in Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant (IFA) as shown in table (2).

Table (2): Immunization protocol

Extraction and purification of the prepared antisera:

Procedure	Protocol Day	Description
Control Serum Collection	Day 0	Pre-immune bleed (5mL per rabbit)
Primary Injection	Day 1	Immunize with 10 ug of antigen in 1 ml CFA, 10 SC sites
1st Booster	Day 14	Boost with 10 ug of antigen in 1 ml IFA, 4 SC sites
2nd Booster	Day 28	Boost with 10 ug of antigen in 1 ml IFA, 4 SC sites
Serum Collection	Day 35	Bleed (~25mL per rabbit)
3rd Booster	Day 42	Boost with 10 ug of antigen in 1 ml IFA, 4 SC sites
Serum Collection	Day 56, 58	Two bleeds (~50mL total per rabbit)
ELISA and Shipping	Day 60	ELISA titration; decide to continue or terminate

After collecting blood, it was allowed to clot for 60 min at 37°C or over night at 4°C. We separated the clot from the sides of the tube (ringing) using a pasteur pipet*t*e and centrifuged at 10000 xg for 10 min at 4°C to separate the serum. Serum was stored at -20°C till used.

Development of ELISA kits and evaluation of its specificity and sensitivity:

The extracted and purified antisera were diluted in phosphate-buffered saline (PBS) (0.1 M, pH 7.4) and were coated (100 µL/well) and incubated overnight at 4 °C. Then, the 96 well plates was washed with PBS then blocked using BSA (5%) and incubated at room temperature for 1 h. Subsequently, the extracted meat protein from cooked reference sample mixtures was added (100 ul/well) and the plates were incubated at 37 °C for ½ h followed by a washing step. Diluted HRP-conjugated anti-pig IgG (1:1000) was added (100 µL/well) and the plate was incubated at 4 °C for 1 h followed by a washing step. As a final step, H2O2/TMB (200 µL/well) liquid substrate system for ELISA (1/20 diluted in citrate buffer pH 5.0) was added to each well and was incubated for 15 min in the dark. Sulfuric acid 0.1 M (50 µL/well) was used to stop the enzymatic reaction and the absorbance was measured at 450 nm using an ELISA plate reader (Biotek).

RESULTS AND DISCUSSION

Identification of unique peptide markers:

We used bioinformatics to compare amino acid sequences across a range of relevant meat animal species to find heat-stable, species-specific peptides as a unique peptide marker for beef, pork, horse, sheep, and chicken. Our preliminary selection was shown in table (3). Based on our selection criteria previously mentioned, we construct a unique peptide marker for each species to develop antisera against each (table 4).

Table (3): Species-specific peptides

Species	Protein	Species specific peptides
Beef	Myosin	EASGPINFTVFLNMFGEK
		HPSDFGADAQAAMSK
		ALEDQLSELK
		KPELIEMLLITTNPYDYAYVSQGEITVPSIDDQEELIATDSAIDI
		LGFTSDER
		LELQSALEEAEASLEQEEGK
		TLALLFSGPASGEAEGGPK
		LLSHSLLVTLASHLPSDFTPAVHASLDK
		MSDEEVEHVEEEYEEEEEAQEEEEVQEEEKPRPR
		LQDAEEHVEVVNAK
Pork	Myosin	GHHEAELTPLAQSHATK
		KPELIELLLITTNPFDYPFISQGEILVASIDDAEELLATDSAIDILGFTPEE
		K
		TLAFLFSGAQTGEAEAGGTK
		TLAFLFTGAAGADAEAGGGK
		ELWDALYQLEIDK
		VGGQAGAHGAEALER
		TLAFLFAER
		LELDDLAGNVESVSK
		ASLLQAEVEELR
		SMSELTMQK
		DFESSQLQSK
		ATTDAAMMAEELK
		ITGGWQMEEADDWLR
		TSEAMPKPHSDAGTAFIQTQQLHAAMADTFLEHMCR
		FFFVSSGPK
		EIYSENSVFIR
		IGQATVASGIPAGWMGLDCGPESSK
		WGDAGATYVVESTGVFTTMEK
		SALAHAVQSSR
Horse	Myosin	GLSDGEWQQVLNVWGK
		VEADIAGHGQEVLIR
		VEADIAGHGQEVLIR
		VVETMQTMLDAEIR
		MSDEEVEHVEEQYEEEEAQEEEAAPPP
		AEVHEEVHEVHVQEEVQEDTAEEER
		TLALLFSGAQTADAEAGGVK
		TLALLFSGPASADAEAGGK
		DLEGEVESEQK
		LETDISQLQGEMEDIVQEAHNAEEK
		GKPEAHFSLIHYAGTVDYNITGWLDK
		EDQCFPMNPPK
		ALGTNPTNAEIK
		HGTVVLTALGGILK
		HPGDFGADAQGAMTK
		DFEHSQLQSK
		LETDVTQLQSEVEDASR
		ARPEFMLPVHFYGHVEHTSHGVEWVDTQVVLAMPYDTPVPGYR
		IAQAVQK
		DLEGEVESEQK
		GKPEAHFSLIHYAGTVDYNITGWLDK
		LETDISQLQGEMEDIVQEAHNAEEK

		LVNDLTGQR HPGDFGADAQGAMTK
Sheep	Myosin	TLAFLFSGAASAEAEGGGAK QGGVGEQLDNLQR WAGGLALSRPELPPHTDTR ARPCCFK RPVGPDAHAEHEDWEDQAGPPR GSAGAQQAR MASRPGHR ALPPPGPQPQLTAPK HGNTVLTALGGILEK VEAGVAGHGQEVLIR LLPPQNTSLIHTK TYCFVADSK EQDTSAHLGR AEEEINAELTAK SYHIFYQILSNR GDIVVVLTGWRPGSGFTNTMR
Chicken	Myosin	LSVEALNSLEGEFK LAMQEFMVLPVGAASFHDAMR KPELIEMLLITTNPYDYHYVSQGEITVPSINDQEELMATDSAIDILGFTP DEK TLALLFANYGGAEAEASAGAGK IPNPTAIPEGQFIDSR NALAHALQSAQHDCDLLR VIQYFASIAAIGHR AGNGVTVTTEMGETLTVPEADVHPQNPPK LELDDVNSNTEQLIK EEQAEPDGTEDCDK LLASLFSNYAGADAGGDGGK EEQAEPDGTEDCDK ILQESHQQALDDLQAEEDK GLCFVPHPQLEFIR GLCFVPHPQLEFIR APEGPRPTPAGDTR NALAHALQSAQHDCDLLR TLALLFASAGGEAESGGGGK NLTEEMAALDENIAK MSDTEEVEHGEAHEAEEVHEEEVHEPAPPPEEKPR LSVEALNSLEGEFK LAMQEFMVLPVGAASFHDAMR ACANPANG DQGTFEDFVEGLR TLALLFATYGGEAEGGGGK NLTEEMAVLDETIAK GALEQTER KVAEQELLDATER VAEQELLDATER ELEGEVDSEQK GADPEDVIMGAFK SFLEELLTTQCDR

Table (4): Selected unique peptide markers to develop specific antisera against each

Species	Protein	Unique peptide markers
Beef	Myosin	LELQSALEEAEASLEQEEGK
		TLALLFSGPASGEAEGGPK
Pork	Myosin	TLAFLFSGAQTGEAEAGGTK
		TLAFLFTGAAGADAEAGGGK
Horse	Myosin	TLALLFSGAQTADAEAGGVK
		TLALLFSGPASADAEAGGK
Sheep	Myosin	WAGGLALSRPELPPHTDTR
		TLAFLFSGAASAEAEGGGAK
Chicken	Myosin	LLASLFSNYAGADAGGDGGK
		NALAHALQSAQHDCDLLR

Specificity and Sensitivity:

As shown in Table (5 & 6), the developed ELISA kits showed 100% specificity during cooked reference sample mixtures testing, with no cross-reactivity detected for the non-target species in the beef/Pork, beef/horse, beef/sheep and beef/chicken mixtures.

In terms of sensitivity, the developed ELISA kits was able to consistently detect pork in the sample mixtures and horse at levels down to 5% w/w (Table 5; Sample 10 & 11). Although sheep and chicken was detected at levels as low as 1% w/w (Sample 16 & 17), this result was found with all the duplicate samples. The beef-specific ELISA test showed greater sensitivity.

Table (5): Results of meat species identification testing in cooked reference sample mixtures.

Sample no.	Sample mixture	Beef	Pork	Horse	Sheep	Chicken
1	Beef 100%	+/+	-/-	-/-	-/-	-/-
2	Pork 100%	-/-	+/+	-/-	-/-	-/-
3	Horse 100%	-/-	-/-	+/+	-/-	-/-
4	Sheep 100%	-/-	-/-	-/-	+/+	-/-
5	Chicken 100%	-/-	-/-	-/-	-/-	+/+
6	Beef 90%/Pork 10%	+/+	+/+	-/-	-/-	-/-
7	Beef 90 %/horse 10%	+/+	-/-	+/+	-/-	-/-
8	Beef 90 %/sheep 10%	+/+	-/-	-/-	+/+	-/-
9	Beef 90 %/chicken 10%	+/+	-/-	-/-	-/-	+/+
10	Beef 95%/Pork 5%	+/+	+/+	-/-	-/-	-/-
11	Beef 95 %/horse 5%	+/+	-/-	+/+	-/-	-/-
12	Beef 95 %/sheep 5%	+/+	-/-	-/-	+/+	-/-
13	Beef 95 %/chicken 5%	+/+	-/-	-/-	-/-	+/+
14	Beef 99%/Pork 1%	+/+	-/-	-/-	-/-	-/-
15	Beef 99%/horse 1%	+/+	-/-	-/-	-/-	-/-
16	Beef 99 %/sheep 1%	+/+	-/-	-/-	+/+	-/-
17	Beef 99 %/chicken 1%	+/+	-/-	-/-	-/-	+/+
18	Beef 99.5%/Pork 0.5%	+/+	-/-	-/-	-/-	-/-
19	Beef 99.5%/horse 0.5%	+/+	-/-	-/-	-/-	-/-
20	Beef 99.5%/sheep 0.5%	+/+	-/-	-/-	-/-	-/-
21	Beef 99.5%/chicken 0.5%	+/+	-/-	-/-	-/-	-/-

The results of developed ELISA kits are reported as positive (+) or negative (-) for each duplicate sample

Table (6): the detection of beef, pork, horse, sheep and chicken in cooked reference sample mixtures, based on observations from the current study

Characteristic	Beef	Pork	Horse	Sheep	Chicken
Sensitivity*	0.5%	5%	5%	1%	1%
Specificity with reference samples	100%	100%	100%	100%	100%
Agreement among duplicate samples**	100%	100%	100%	100%	100%

*****Lowest consistent detection level (w/w) in a binary mixture of pork and beef.
**Percentages are based on a total of 21 samples tested in duplicate with each assay.

Discussion:

Meat and meat-based products constitute a significant percentage of the human diet worldwide. Beef is the top red meats consumed. The contribution of beef to the global food trade id is 59.0 million tons of beef and veal that expected to be produced globally (USDA, 2016). Meat species may be easy identifiable when sold as whole cuts; however, processing conditions and techniques may change the texture, flavor, and color of meat, making it difficult to authenticate species in food products containing processed meats (Cawthorn et al., 2013). The inability to readily identify meat species in processed products gives rise to the potential for species mislabeling, in which one species is substituted either partially or completely for another species. In most cases, species mislabeling is a kind of economically motivated adulteration (EMA), in which a product is intentionally mislabeled for reasons of economic gain (FDA, 2009). Due to price differences among meat species, there are economic incentives accompanied with meat species mislabeling. For example, the average retail value of beef in 2015 was US$13.31/kg while the average retail value of pork for the same year was US$8.49 (USDA, 2016), resulting in an economic incentive for the substitution of pork for beef in a processed product. Substitution can occur at any point in the supply chain, from the slaughterhouse up until the point of sale (Premanandh, 2013). In addition to meat products may be mislabeled due to cross-contamination when processing different types of meat on the same equipment, as has been suggested in previous

Mislabeling of meat species in processed foods has a number of potentially detrimental effects such as the exposure of consumers to meat allergen risks, infringement of religious practices, and economic deception (Ballin, 2010; Kane & Hellberg, 2016; Okuma & Hellberg, 2015). A number of studies have detected undeclared species in processed meat products such as deli meats, minced meats, burger patties, sausage and canned meats (Ayaz et al., 2006; Cawthorn et al., 2013; Di Pinto et al., 2015; Flores-Munguia et al., 2000; Okuma & Hellberg, 2015). Some of the most commonly undeclared species within these products were beef, pork, poultry, and sheep. These findings are concerning from a religious point of view, as some religions prohibit the consumption of beef and/or pork (Sattar et al., 2004). In addition to the studies mentioned above, horsemeat was detected as an undeclared ingredient in numerous beef products in the 2013 horse gate scandal in Europe (National Audit Office, 2013). Adulteration and misbranding of meat products is prohibited globally. Adulteration can occur when an ingredient has been completely or partially omitted, and/or if any ingredient has been substituted within a meat product. A misbranded product includes one whose labeling is false or misleading or if it is offered for sale under the name of another food.

To determine if meat species have been partially or completely substituted for cheaper alternatives in processed food products, DNA or protein-based methods are often used (Ballin, 2010). Two of the most commonly used methods for this purpose are enzyme-linked immunosorbent assay (ELISA), a protein-based method, and real-time polymerase chain reaction (PCR), a DNA based assay. Most of the regulatory bodies rely on ELISA method for

identifying animal species in cooked and canned meat (USDA, 2005). ELISA technique is recognized as being sensitive and robust and it has been used in several studies for detecting low levels of a target species within mixtures (Yamamoto et al., 2015). Therefore, the objective of this study was to develop ELISA kits for detection of meat species adulteration and evaluate the developed kits in detection of meat species in cooked reference sample mixtures.

This work has identified muscle proteins that are able to persist in meat preparations that have undergone heavy processing. Bioinformatics analysis enabled us to obtain an initial list of peptides unique to beef, pork, horse, sheep and chicken. The main focus of this study was the identification of unique peptide markers for the five species to develop specific antisera for each species with no cross reactivity with others. Our preliminary selection has shown in table (3). Based on our selection criteria previously mentioned, we construct unique peptide marker for each species to develop antisera against each (table 4).

The developed ELISA kits showed 100% specificity during cooked reference sample mixtures testing, with no cross-reactivity detected for the non-target species in the beef/Pork, beef/horse, beef/sheep and beef/chicken mixtures. In terms of sensitivity, the developed ELISA kits were able to consistently detect pork in the sample mixtures and horse at levels down to 5% w/w (Table 5; Sample 10 & 11). Although sheep and chicken was detected at levels as low as 1% w/w (Sample 16 & 17), this result was found with all the duplicate samples.

The beef-specific ELISA test showed greater sensitivity. Our results are matched with previous studies using ELISA assay with monoclonal antibodies (mAbs) that have reported lower detection limits for different meat species. For example, studies using a sandwich ELISA with porcine-specific mAbs have reported the ability to detect pork at levels of 0.05 - 0.5% w/w in various meat mixtures (Chen & Hsieh, 2000; Liu et al., 2006). Similarly, Yamamoto et al. (2015) were able to detect beef at levels of 0.1% w/w in a beef and pork meal mixture using a sandwich ELISA with two bovine-specific mAbs. Future studies are still required for validation of the developed kits and comparing its results with the commercially used kits before entry into the production phase.

CONCLUSION

Overall, the developed ELISA kits in this study was a specific and promising tool to detect meat species in the cooked reference sample mixtures and sensitive method for beef, followed by sheep and chicken, followed by pork and horse species. The results of this study also suggest that ELISA was found to be low time consuming and easy to perform. Further studies are still required for validation of the developed kits and comparing its results with the commercially used kits before entry into the production phase.

ACKNOWLEDGEMENTS

The authors are grateful to the General Organization of Veterinary Services, Egypt for supplying them with meat species standard samples and Hangzhou Dangang Biological Technology Co., Ltd., China for bioinformatics support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

For conducting this study, no funds were received.

REFERENCES

Ayaz, Y., Ayaz, N. D., & Erol, I. (2006). Detection of species in meat and meat products using enzyme-linked immunosorbent assay. *Journal of Muscle Foods*, 17(2), 214 - 220. https://doi.org/10.1111/j.1745-4573.2006.00046.x

Ballin, N. Z. (2010). Authentication of meat and meat products. *Meat Science*, 86(3), 577 – 587. https://doi.org/10.1016/j.meatsci.2010.06.001

Cawthorn, D.-M., Steinman, H. A., & Hoffman, L. C. (2013). A high incidence of species substitution and mislabelling detected in meat products sold in South Africa. *Food Control*, 32(2), 440 - 449. https://doi.org/10.1016/j.foodcont.2013.01.008

Chen, F. C., & Hsieh, Y. H. (2000). Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. *Journal of AOAC International*, *83*(1), 79–85. http://dx.doi.org/10.1093/jaoac/83.1.79

Di Pinto, A., Bottaro, M., Bonerba, E., Bozzo, G., Ceci, E., Marchetti, P., et al. (2015). Occurrence of mislabeling in meat products using DNA-based assay. *Journal of Food Science and Technology*, 52(4), 2479 - 2484. https://doi.org/10.1007/s13197-014-1552-y

Flores-Munguia, M. E., Bermudez-Almada, M. C., & Vazquez-Moreno, L. (2000). Detection of adulteration in processed traditional meat products. *Journal of Muscle Foods*, 11(4), 319 - 325. http://dx.doi.org/10.1111/j.1745-4573.2000.tb00435.x

Kane, D., & Hellberg, R. (2016). Identification of species in ground meat products sold on the U.S. commercial market using DNA-based methods. *Food Control*, 59, 158 - 163. https://doi.org/10.1016/j.foodcont.2015.05.020

Liu, L. H., Chen, F. C., Dorsey, J. L., & Hsieh, Y. H. P. (2006). Sensitive monoclonal antibody-based sandwich ELISA for the detection of porcine skeletal muscle in meat and feed products. *Journal of Food Science*, 71(1), M1 - M6. https://doi.org/10.1111/j.1365-2621.2006.tb12393.x

Montowska M, Alexander MR, Tucker GA, Barrett DA. 2014. Rapid detection of peptide markers for authentication purposes in raw and cooked meat using ambient liquid extraction urface analysis mass spectrometry. *Anal Chem*. 86:10257–10265. https://doi.org/10.1021/ac502449w

Okuma, T. A., & Hellberg, R. S. (2015). Identification of meat species in pet foods using a real-time polymerase chain reaction (PCR) assay. *Food Control*, 50, 9 - 17. http://dx.doi.org/10.1016/j.foodcont.2014.08.017

O'Mahony PJ. 2013. Finding horse meat in beef products—a global problem. *QJM*. 106:595–597. https://doi.org/10.1093/qjmed/hct087

Premanandh, J. (2013). Horse meat scandal - A wake-up call for regulatory authorities. *Food Control*, 34(2), 568 – 569. https://doi.org/10.1016/j.foodcont.2013.05.033

Şakalar, E., Abasiyanik, M. F., Bektik, E., & Tayyrov, A. (2012). Effect of heat processing on DNA quantification of meat species. *Journal of food science*, *77*(9), N40–N44. https://doi.org/10.1111/j.1750-3841.2012.02853.x

Sattar, S. P., Ahmed, M. S., Madison, J., Olsen, D. R., Bhatia, S. C., Ellahi, S., Majeed, F., Ramaswamy, S., Petty, F., & Wilson, D. R. (2004). Patient and physician attitudes to using medications with religiously forbidden ingredients. *The Annals of pharmacotherapy*, *38*(11), 1830–1835. https://doi.org/10.1345/aph.1E001

Yamamoto, T., Kato, M., Endo, K., Kotoura, S., & Takeda, Z. (2016). Detection of ruminant meat and bone meal in feeds by sandwich ELISA with monoclonal antibodies. *The Journal of veterinary medical science*, *77*(12), 1605–1609. https://doi.org/10.1292/jvms.15-0078