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DNA Quality and Integrity of Nuclear and Mitochondrial Sequences from Beef Meat as Affected by Different Cooking Methods

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Summary

The extraction of high quality DNA from processed meat can often represent the crucial step in an authentication process by PCR-based methods. In this study, the effect of three different domestic cooking methods (roasting, boiling, and microwave) on DNA isolated from two beef muscles has been investigated. The quality of extracted DNA was evaluated by amplifying target sequences from mitochondrial and nuclear genes, as well as by monitoring the yield, purity, and degradation of the extracted DNA. Large PCR fragments (length >900 bp) were successfully amplified from both genes in all samples. The cooking methods caused significant differences in terms of quality and quantity of DNA recovered from meat.

Key words: beef, cooking method, DNA, species identification, PCR

Introduction

The increasing awareness of consumers regarding the composition of food products has led food technologists to develop many techniques for detection and identification of the ingredients used in manufactured foods. Of these techniques, DNA-based methods have been widely applied for species identification of animal tissues in meat products, since DNA, despite suffering some alterations, offers the advantage of being more resistant than proteins to different treatments during food processing (*1–3*). Most DNA-based methods for species identification in food consist of the highly specific amplification of one or more DNA fragments by means of polymerase chain reaction (PCR), which has a great potential due to its speed, simplicity, sensibility and specificity (*4–6*). PCR amplification often involves target sequences in mitochondrial DNA (*7–14*) rather than in nuclear DNA (*15–17*). Mitochondrial DNA (mtDNA) is preferred over nuclear DNA (nDNA) essentially because of its maternal inheritance, more conservative sequence in different species of animals and the high copy number per cell (*3,13,18–20*). However, Schultz *et al*. (*21*) reported that the mitochondrial content may vary widely according to the tested tissue, which may confound attempts to develop quantitative species-specific tests. Therefore, several nuclear genes, which are conserved throughout the vertebrates and invertebrates, like actin and histones, have been successfully targeted for addressing adulteration and conservation issues (*22–24*). Nevertheless, successful PCR amplifications of mtDNA and nDNA regions depend on purity and quality of DNA template, so that the heating process chosen for cooking meat can have a great impact on the final result. Previous studies have shown that DNA can be broken down to smaller pieces in meat that is cooked at high temperatures (*11,15,25,26*), so that the amplification of shorter DNA fragments is recommended (*27*). In this paper, the effects of three domestic cooking methods on the quality and quantity of DNA extracted from two beef muscles are evaluated, as well as the integrity of target sequences of mitochondrial and nuclear DNA.

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Materials and Methods

Meat samples

Two muscles, *extensor carpi radialis* (ECR, foreleg) and *supraspinatus* (SS, chuck), were removed at 24 h *post mortem* from 10-month-old cattle. Each muscle was trimmed of external fat and epimysial connective tissue, and cut into steaks perpendicular to the direction of the fibre. Meat was cut into small, 3×3×1.85 cm (length×width×height) pieces, which were collected and stored at –40 °C until cooking treatments.

Cooking treatments

Meat pieces of each muscle were grouped according to the following cooking methods: roasting (RO), boiling (BO) and microwave (MW). One group of meat pieces was used as raw control (RA). After the treatments, the endpoint core temperature of the meat samples was assessed using a thermocouple probe (HI 9025, Hanna Instruments, Woonsocket, RI, USA) inserted into the meat. The main characteristics of each method are shown in Table 1. Cooking time in all treatments was 5 min, the period previously verified as sufficient for meat consumption in terms of overall acceptance by a small consumer panel (N=10) of laboratory staff. After cooking treatment, meat was cooled to room temperature, surface-dried with a filter paper, and used for analysis.

Table 1. Main characteristics of cooking methods used in this study

Cooking methods	Description	Endpoint core temperature*/°C
RA	Meat was not subjected to any cooking method	–
RO	Samples were cooked on a preheated grill (300 °C) turning halfway through the cooking period	73.7±2.2
BO	Meat samples were placed directly into the boiling water (100 °C)	75.1±3.3
MW	Meat was placed in a glass dish and cooked in a domestic microwave oven (DēLonghi mod. 500 MW, Treviso, Italy) at 560 W	65.9±2.2

RA=raw meat, RO=roasting, BO=boiling, MW=microwave
*mean values of 3 determinations±standard deviation

DNA extraction

Meat samples were ground in liquid nitrogen with mortar and pestle and the resultant powder was used to isolate DNA according to the method described by Sambrook *et al*. (*28*), with some modifications. Briefly, about 300 mg of powdered tissue were mixed with 5 mL of extraction buffer (100 mM Tris-HCl, pH=9.0, 100 mM NaCl, 5 mM EDTA, 1 % SDS, 30 μL proteinase K (10 mg/mL)) and incubated at 65 °C for 2 h. The above mixture was extracted twice with an equal volume of phenol/chloroform (1:1) and twice with an equal volume of chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated with isopropanol and the RNA was degraded by 100 μg/mL Ribonuclease A (Sigma-Aldrich, Inc., St. Louis, MO, USA) for 1 h at 37 °C. DNA was then purified by successive double extractions with phenol/chloroform and chloroform/isoamyl alcohol, respectively, and after that precipitated with ice-cold absolute isopropanol and washed twice with 70 % ethanol. The pellet was finally dried and resuspended in 100 μL of ddH_2O. The obtained DNA solutions (N=30) from the subsamples of each treatment were used to evaluate yield, purity and integrity of DNA.

DNA quality

The integrity of the isolated DNA was checked on a 1 % (by mass per volume) agarose gel under ultraviolet light, after staining with ethidium bromide (0.5–1 μg/mL). A 1-kb DNA ladder (New England Biolabs, Inc., Hitchin, Hertfordshire, UK) was used as a marker.

The DNA yield (expressed as μg per g of tissue) was calculated using a UV/VIS spectrophotometer (Model ND--1000 NanoDrop, Wilmington, DE, USA) and the $A_{260\ nm}/A_{280\ nm}$ absorption ratio was taken as purity index.

Primer design

Cytochrome b (*cytb*) and cathepsin B (*CTSB*) genes were selected for evaluating the quality of the extracted mitochondrial (mtDNA) and nuclear (nDNA) DNA, respectively. Sequences for both genes were obtained from GenBank® (NCBI, Bethesda, MD, USA). Primer positions were chosen to give amplicon sizes between 800 and 1000 bp using the software program Primer3 (*29*). Primer sequences (Table 2) were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Table 2. Primer sequences and PCR conditions

Gene	Fragment length	Primer sequence (5′→3′)	PCR protocol*
	bp		
Cytb	915	F: TCGACCTTCCAGCCCCATCG	94 °C 30 s,
		R: GGCATTGGCTGAGCAGTCGGA	64 °C 45 s,
			72 °C 90 s
CTSB	914	F: ACGAACACGTAGACACACAA	94 °C 30 s,
		R: GTATGGCTGGTAAAAAGACG	55 °C 45 s,
			72 °C 90 s

Cytb=Bos taurus mitochondrial gene for cytochrome b (GenBank® accession no. D34635), *CTSB=Bos taurus* cathepsin B (GenBank® accession no. NM174031)

*The PCR amplifications were performed with an initial denaturation at 94 °C for 4 min and with a final extension at 72 °C for 10 min. The cycles (N=35) of denaturation, annealing and extension for each amplicon are indicated

PCR amplification

PCRs were performed using a peqSTAR Thermocycler Universal 96 (PEQLAB GmbH, Erlangen, Germany) in a final volume of 50 μL containing 0.1 μg of DNA template, 20 mM Optimized DyNAzyme™ Buffer, 0.2 mM

dNTPs, 0.4 mM of each primer and 2.5 units of DyNAzyme™ II DNA Polymerase (Finnzymes OY, Riihitontuntie, Espoo, Finland).

The amplification products were resolved by electrophoresis on a 1.5 % (by mass per volume) agarose gel stained with ethidium bromide (0.5–1 μg/mL) and visualized under UV light. Bands of appropriate sizes were identified by comparison with a 100-bp ladder (New England Biolabs Ltd, Hitchin, Hertfordshire, UK).

Data analysis

The effects of cooking methods and muscle type on DNA yield (μg/g) and purity ($A_{260\ nm}/A_{280\ nm}$ ratio) were assessed by a two-way analysis of variance (ANOVA) with a data set consisting of 30 cases (*i.e.* DNA solutions) per treatment. When the difference in the ANOVA was statistically significant, pairwise comparisons of the above parameters were analysed by Tukey's honestly significant difference test (HSD) at $p \leq 0.05$. The presence and absence of bands, visualized under UV on agarose gels used to check PCR products, was scored (1, 0, respectively), and the data were expressed as positive cases of DNA band presence in relation to total cases. The entire experiment was repeated twice.

Results and Discussion

The quality of the extracted DNA

The main characteristics of DNA extracted from raw and cooked meat are reported in Table 3. The ANOVA showed a significant effect of cooking methods on DNA yield ($p<0.001$), whereas muscle type was not significant. The amount of DNA extracted from raw meat was about 490 μg/g, somewhat more than 357 μg/g obtained by Zhang *et al*. (*30*). However, except for the boiled samples, the amount of DNA extracted from raw meat was significantly lower than that of cooked meat. This difference may be attributed to the disruption of nuclear or cellular membranes by heating processes, which allowed more DNA to be released from the individual muscle cells. The disruption of cell membranes, as well as other meat structural changes (shrinkage of meat fibres, the aggregation and gel formation of myofibrilar and sarcoplasmic proteins, shrinkage and solubilisation of the connective tissue, *etc*.), are due to the denaturation of the different meat proteins during heating (*31–34*). However, membrane disruption and, consequently, DNA recovery are strongly related to the heat transfer efficiency of each cooking method, as well as to the cooking time. For boiled meat (endpoint core temperature >75 °C, Table 1), the better heat transfer efficiency led to a rapid membrane disruption and to a DNA denaturation, resulting in lower yield compared to raw meat. Previous studies indicated that DNA extracted from raw meat was significantly longer than that obtained from meat cooked in a water bath with temperatures ranging from 75 to 100 °C (*15*). In this study, the greatest DNA yield was obtained from microwaved samples: for both muscles, the amount of DNA extracted was more than 1 mg/g. However, gel electrophoresis analysis revealed that DNA extracted from microwaved meat was partially degraded, whereas intact genomic DNA bands were observed from raw and other cooked meat samples (Figs. 1a and b). Denaturation of double-stranded to single-stranded DNA causes an increase in absorbance (at 260 nm) of DNA solution. This phenomenon, called hyperchromic effect (*35*,*36*), may be in part responsible for the increase in DNA yield from microwaved meat.

Cooking methods also significantly ($p<0.001$) affected DNA purity (Table 3). For all samples (raw and cooked meat), the ratio of absorbance ($A_{260\ nm}/A_{280\ nm}$) ranged from 1.810 to 1.857 for ECR, and from 1.812 to 1.858 for SS, respectively. These results indicate a successful removal of contaminating molecules (*28*). For raw meat, the resulting ratio (1.820 and 1.823 for ECR and SS, respectively) was higher than that (1.73) reported by Zhang *et al*. (*30*), but lower than that (1.969) reported by Aslan *et al*. (*15*). These authors, however, found reduced $A_{260\ nm}/A_{280\ nm}$ absorbance ratios in cooked meat compared with raw meat. The present results show that, for both muscle types, DNA purity of raw samples was significantly higher than that of roasted samples, but lower than that

Table 3. Yield, purity and integrity of DNA extracted from raw and cooked meat (mean values±S.D.)

DNA parameter		Yield/(μg/g)	Purity $A_{260\ nm}/A_{280\ nm}$	Integrity*	
				Cytb	*CTSB*
extensor carpi radialis	RA	$(490\pm13)^{a}$	$(1.820\pm0.007)^{a}$	30/30	30/30
	RO	$(883\pm13)^{b}$	$(1.810\pm0.008)^{c}$	30/30	30/30
	BO	$(285\pm13)^{c}$	$(1.826\pm0.009)^{ab}$	30/30	30/30
	MW	$(1072\pm13)^{d}$	$(1.857\pm0.008)^{d}$	30#/30	30#/30
supraspinatus	RA	$(492\pm13)^{a}$	$(1.822\pm0.007)^{ab}$	30/30	30/30
	RO	$(884\pm14)^{b}$	$(1.812\pm0.009)^{c}$	30/30	30/30
	BO	$(286\pm12)^{c}$	$(1.828\pm0.009)^{b}$	30/30	30/30
	MW	$(1073\pm12)^{d}$	$(1.858\pm0.008)^{d}$	30#/30	30#/30
significance	CM	***	***		
	M	NS	NS		

RA=raw meat, RO=roasting, BO=boiling, MW=microwave, CM=cooking methods, M=muscle, NS=not significant, ***$p<0.001$
*numbers shown are positive cases/total cases
#fainter bands were observed
[a-d]mean values with different superscripts differ significantly ($p<0.05$) as measured by Tukey's HSD test

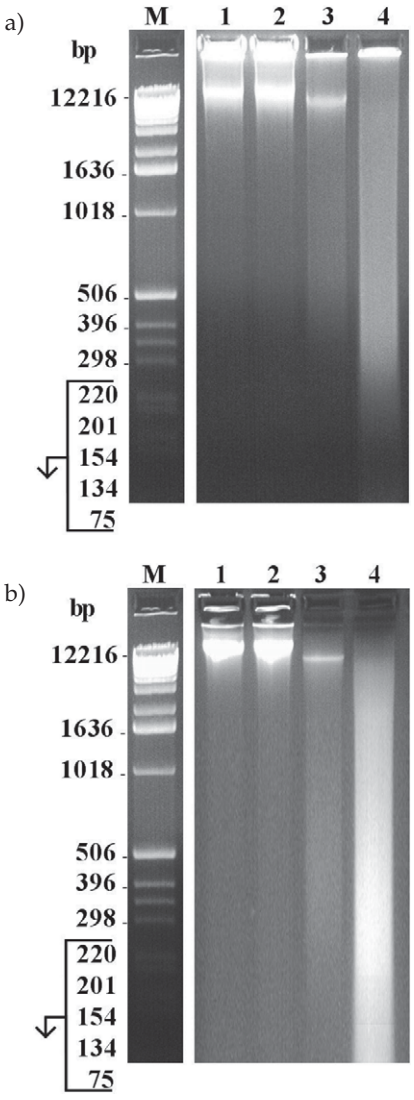

Fig. 1. A representative gel electrophoregram showing the effects of different cooking methods on the DNA extracted from: a) bovine *extensor carpi radialis* and b) *supraspinatus*. In both gel agarose (1 %) electrophoretic images lanes 1–4 include samples of raw (1, RA), roasted (2, RO), boiled (3, BO) and microwaved (4, MW) meat. M, 1-kb DNA ladder

of microwaved ones. No difference was observed between the raw and boiled samples. The highest $A_{260\,nm}/A_{280\,nm}$ absorbance ratio was observed for the DNA obtained from microwaved meat. This result, however, is probably due to the increase in the absorbance of ultraviolet light of partially denatured DNA solutions (*i.e.* hyperchromic effect).

PCR amplification of the extracted DNA

DNA samples extracted from all raw and cooked samples were subjected to amplification by PCR under uniform conditions. PCR amplifications were successful in all samples: both mtDNA and nDNA regions, in fact, were amplified (Table 3). All cooking methods allowed the detection of large PCR fragments (length >900 bp) for both genes, revealing that long DNA molecules were still intact after 5 min of cooking time. Evidently, the degradation observed in microwave samples (endpoint core temperature <66 °C, Table 1) was not so severe to compromise the PCR amplifications. Previous studies (*26*) reported that the fragment size of the DNA from the meat samples which have been heated to 100 °C and above was reduced from approx. 1100 to approx. 300 bp. Similarly, Meyer *et al*. (*37*) showed that the average size of DNA fragments extracted from meat autoclaved at 121 °C for 10 to 60 min was 300 bp. Matsunaga *et al*. (*11*) reported that horse meat could not be determined after autoclaving at 120 °C for 30 min by amplification of a 439-bp mitochondrial DNA region. Arslan *et al*. (*25*) reported that a 271-bp fragment of the mtDNA was successfully amplified from meat cooked by different methods (boiling, roasting and pressure cooking) with the exception of pan frying. These results indicated that DNA integrity is strictly related to the heat transfer efficiency and, hence, to the core temperature of the cooked samples. Most recently, Aslan *et al*. (*15*) showed that large PCR products (between 800 and 1000 bp) of three nuclear genes were observed for samples cooked to internal temperatures ranging from 75 to 90 °C, but above these temperatures, no PCR products greater than 800 bp could be observed.

The present results from agarose gel electrophoresis showed that the band for microwaved meat was fainter than those of other (raw and cooked) meat samples (Figs. 2a and b, 3a and b). This different band intensity, which was evident for both muscles tested, indicated that DNA fragmentation occurred when meat was cooked in a microwave. In this case, the absorbance (*A*) values nominally increased as the magnitude of DNA denaturation

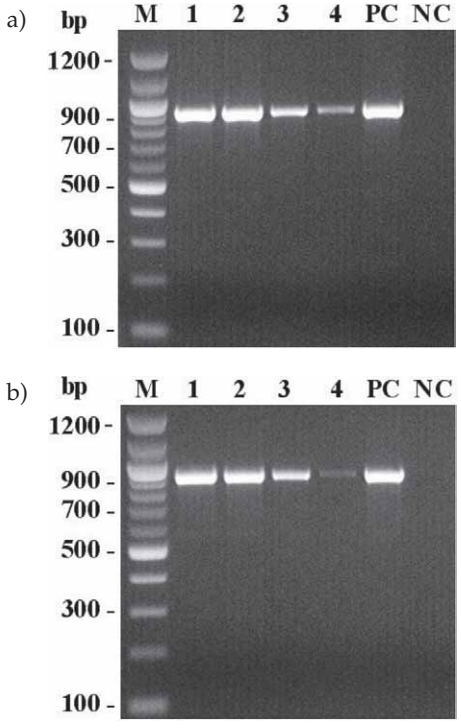

Fig. 2. A representative gel electrophoregram showing PCR amplifications of DNA extracted from bovine *extensor carpi radialis* subjected to different cooking methods using: a) *cytb* and b) *CTSB* primers. In both gel agarose (1.5 %) electrophoretic images lanes 1–4 include samples of raw (1, RA), roasted (2, RO), boiled (3, BO) and microwaved (4, MW) meat. M, 100-bp DNA ladder; PC, DNA extracted previously from meat and used as positive control; NC, negative control without DNA

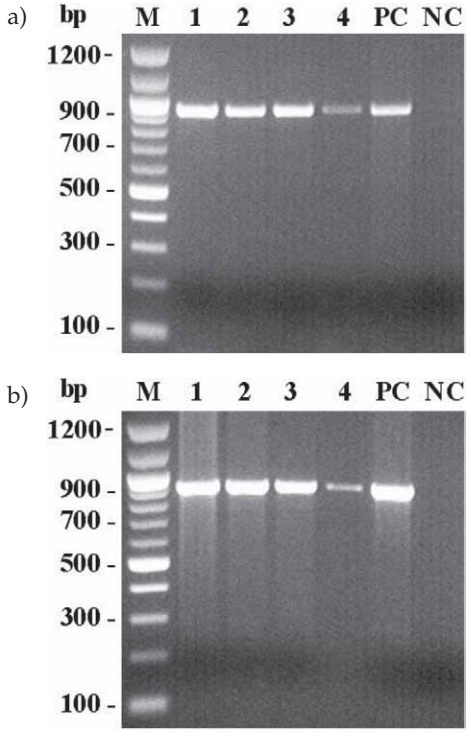

Fig. 3. PCR amplifications of DNA extracted from bovine *supraspinatus* subjected to different cooking methods using: a) *cytb* and b) *CTSB* primers. In both gel agarose (1.5 %) electrophoretic images lanes 1–4 include samples of raw (1, RA), roasted (2, RO), boiled (3, BO) and microwaved (4, MW) meat. M, 100-bp DNA ladder; PC, DNA extracted previously from meat and used as positive control; NC, negative control without DNA

increased, so that the UV spectrophotometric method overestimated the DNA yield of microwaved samples. As a result, the available amount of template DNA for PCR amplification was lower than the amount isolated from other meat samples, even though the *A* values were equal. Our findings are consistent with those of Yang and Speller (*38*), who found that if DNA is heavily degraded, the longer PCR fragment will be less favourably amplified, resulting in weaker bands, while those samples with a better DNA preservation would show stronger amplification of the longer fragments. Another interesting result is that the band intensity for microwaved meat was much weaker when amplifying target sequences from *CTSB* gene compared to *cytb* gene, thus indicating that nuclear DNA suffered more fragmentation than mitochondrial DNA. This result may also be related to the fact that mitochondrial DNA is more abundant than nuclear DNA (*3,13,18,20*), so that it is more reliable to amplify mitochondrial genes from degraded DNA samples because of their greater copy number.

Conclusion

The effects of several heat treatments on meat quality characteristics (*i.e.* fat content, protein and lipid fractions, Warner-Bratzler shear force, cooking loss, colour, texture, *etc*.) are well documented (*39–45*). Lawrence *et al*. (*46*) showed that the effects of cooking methods may vary widely according to the specific characteristic of each muscle type. The results of this study show that the domestic heating procedures commonly used for cooking meat can significantly affect the yield and also the quality of the DNA extracted from meat, although no significant differences were observed between the tested muscles. Five minutes of cooking time, the period verified as acceptable for meat consumption in terms of sensory properties, seems to disrupt cell membranes and thus lead to isolation of more DNA from cooked than raw meat. For boiled meat, however, a better heat transfer efficiency (and possibly loss by diffusion into boiling water) resulted in lower DNA yield compared to raw and cooked meat. Among cooking methods, microwave heating produced the highest DNA yield, more than 1 mg per g of tissue. However, this result, determined by UV absorbance, might have been an overestimation due to hypercromic effect. Cooking methods had significant effect on DNA purity, with the microwaved samples showing the highest value. The samples from raw and cooked meat also had good $A_{260\,nm}/A_{280\,nm}$ ratios, thus indicating the absence of contaminating compounds. The resulting differences among cooking methods in terms of DNA yield and purity were in line with the results of PCR amplifications. Large fragments (>900 bp) of mitochondrial and nuclear regions were successfully amplified from DNA isolated from raw and cooked meat, thus revealing that short cooking procedures did not excessively damage the DNA. Thus, DNA extracted from microwaved meat produced fainter bands in comparison with other templates, and this effect was more pronounced with the nuclear than mitochondrial gene. The conclusion is that the applicability of DNA-based techniques for animal species and subspecies identification can be significantly affected by domestic cooking procedures (heat transfer, time, temperature, *etc*.), so that the genes, as well as the size of the fragment to be amplified by PCR, should be carefully selected.

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