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The effect of heat and pressure processing on the fragmentation of DNA and implications for detection of meat using real-time PCR.

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Abstract

The design of real-time PCR assays for the detection of meat in processed products has focused on using small amplicons often to the detriment of specificity. However, the relationship between amplification rates and amplicon size for processed meat products has yet to be determined. To investigate this relationship real-time PCR assays were designed to give a series of amplicons of increasing size. These assays were then used to assess amplification rates, in relation to amplicon size, in processed meat matrices. It was found that, although the most sensitive assays were those which used the smallest amplicons, amplification was still observed using amplicons of 351 base pairs for highly processed samples. It was found therefore, that although in general, amplicons should be as small as possible, larger amplicons give efficient amplification and that small amplicons should not be chosen if they compromise assay specificity.

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Key words: real-time PCR, heat, pressure, processing, meat, DNA, detection¶

Introduction

There are many reports outlining the detection of meat species in food in the literature. From these reports, DNA appears to be the analyte of choice and real-time polymerase chain reaction (PCR) the most appropriate analytical tool. However, there are few reports examining the design and optimisation of real-time PCR assays, although many studies cite various significant parameters that must be considered during the design process for efficient amplification and therefore sensitive assays.

Deleted: It has long been known that heat and pressure processing fragments DNA and that shorter amplicons allow greater sensitivity of PCR based assays (Meyer Candrian and Luthy 1993). Matsunaga et al. (1999) studied the effect of heat processing on the detection limit of a PCR based system using universal primers. This study used comparatively gentle cooking regimes and found that there was no effect of heating to 65°C, 70°C or 75°C for 30 minutes on the detection of meat in mixtures of meat. More stringent cooking processes were analysed for their effect on the detection of cow in a variety of meat matrices by Brodman and Moor (2003). This study found that canning reduced the sensitivity of the assay ten fold, although the milder cooking regimes did not appear to affect sensitivity. ¶

Amplification efficiency is a measure of how close the amplification rates are to the theoretical doubling of template DNA with each cycle. Amplification rates are affected by many factors amongst which include, the number of target molecules, the sequence of the target, the buffer and thermostable polymerase, the cycling conditions, primer sequence and the presence of inhibitors in the sample. One of the most cited parameters, from the work of Meyer et al., (1993), is amplicon size since this group reported that the average size of DNA fragments following heat and pressure processing was approximately 300 base pairs. Consequently, most systems for the detection of DNA from highly processed matrices used amplicons of approximately 300 base pairs (Tartaglia et al 1998; Frezza et al 2003; Bottero et al

2003) and the need for gel visualisation meant that the amplicons were usually over 130 base pairs.

The advent of real-time PCR has allowed the development of assays which utilise very small amplicons and the size of amplicon has, in some studies, appeared to override all other considerations, compromising assay specificity in some instances (Brodman and Moor 2003; Dooley et al. 2004; Mendoza-Romero et al. 2004).

Brodman and Moor (2003) designed assays for the smallest possible amplicons to ensure amplification of highly processed feeds. However using amplicons of 66 and 76 base pairs there was only intermittent detection of samples of cow bone meal. These primers were also shown to cross amplify red and roe deer, moose, sheep and pig DNA. Similarly ruminant specific assays, using an amplicon of 88 base pairs, were shown to cross amplify with the non-ruminants pig, horse, chicken and turkey (Mendoza-Romero et al. 2004). The interpretation of assays which cross amplify is problematic since it is not clear whether there has been detection of the target or the cross amplifying species.

The objective of the study described here was to extend the current knowledge for the design and optimisation of real-time PCR assays for the detection of meats in food products. We designed experiments to elucidate the effect of amplicon size on the sensitivity of real-time PCR assays when applied to heat and pressure processed matrices. We designed real-time PCR assays for turkey, where one primer remained constant and was paired with a series of alternative primers to give amplicons of increasing size. This primer series was used on raw turkey template DNA to determine the rate of amplification. We went on to study the effect of processing on amplification rates using preparations of meat which had been boiled, baked, canned or autoclaved. Finally we applied the turkey primer series to the detection of turkey in poultry meal to illustrate the effect of amplicon size on the detection of highly processed samples. The data presented here should allow a greater flexibility during the design of real-time PCR assays, leading to protocols for the detection of meats in food products with increased specificity.

Experimental

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Meat samples and processing

Authentic meat samples were sourced from local suppliers and held at 4 °C until processing. Samples were homogenised in a food blender and aliquots processed or left raw. Boiled samples were sealed in plastic pouches and boiled in water for 2 and 32 minutes. Baked samples were cooked in an oven at 180 °C for 2 and 32 minutes. Canned samples were processed in a Mather and Platt vertical retort, at 121 °C for 40 minutes, to achieve a sterilising (F₀) value of at least 7.6 and autoclaved samples were autoclaved at 121 °C for 15 minutes. Raw, boiled, baked and autoclaved samples were frozen at -20 °C and canned samples were stored at room temperature until DNA extraction.

DNA extraction from meat mixtures

DNA was extracted from meat matrices using a modified version of the Wizard DNA extraction Kit (Promega). Briefly, 5 g of each sample was incubated at 65 °C in 10 ml CTAB buffer (2% (w/w) Cetyl trimethyl ammonium bromide (CTAB), 20mM EDTA, 0.1M Tris[hydroxymethyl]aminomethane (tris-base) and 1.4M sodium chloride (pH 8) containing 80 ng/ml proteinase K (Sigma). After 2 hours the samples were vortexed for 10 seconds and centrifuged for 10 min at 4000 g. 1 ml of the supernatant was combined with 800 µl chloroform, vortexed briefly and centrifuged for 10 min at 14000 g. 500 µl of the aqueous phase was combined with 1 ml Wizard DNA resin and incubated for 5 min at room temperature. The DNA was then purified exactly according to the manufacturers' instructions and eluted with 100 µl Tris EDTA buffer previously heated to 65 °C.

Design of TaqMan[®] primer and probe sets

The primers and probe sets were designed using the mitochondrial *cytochrome b* gene for turkey (GenBank Accession L08381) using Primer Express[®] software (version 1.5). The primers were expected to have the following specific qualities: to produce an amplicon at target sizes; to have the 3' end of the primer as close as possible to the probe without overlapping it; to have a T_m between 58 and 60 °C; to contain 20-80% G or C, to be between 9 and 40 bases long, and to have a maximum of 2 G or C bases within the last 5 residues of the 3' end. PrimerExpress[®] design parameters were adapted to achieve primer design. The probe was designed to have a T_m 10 °C higher

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Deleted: DNA was extracted from meat mixtures using a modified version of the GenElute™ Mammalian Genomic DNA Extraction Kit (Sigma). Briefly, 2 g of tissue was finely minced with a scalpel blade, prior to incubation at 65 °C in 8 ml CTAB buffer (0.055 M CTAB, 1.4 M NaCl, 0.1 M Tris and 0.02 M EDTA, pH 8.0) containing 50 µg/ml proteinase K (Sigma). After 1 hour, the samples were cooled briefly and 400 µL of the supernatant used to extract the DNA exactly according to the manufacturer's instructions. The final elution step was repeated once and the eluates pooled. DNA was stored short term at 4 °C or long term at -20 °C.¶

Deleted: DNA extraction from poultry and meal samples¶

DNA was extracted from 5 g samples of meal using the Nucleospin DNA extraction kit available from Nucleon exactly according to manufacturer's instructions.¶

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than that of the primers, to contain between 20 and 80% G or C, to be between 9 and 40 bases long, have a T, C or A at the 5' end and to contain more C's than G's (Oehlmann 1999). Additionally primers were designed to be mismatched at the 3' position to negate spurious amplification with other species of farm animals and humans (Hird, Goodier and Hill 2003). The turkey primers were truncated at the 5' end until specific (Hird et al. 2004). Probes were 5' labelled with 6-carboxyfluorescein (FAM) and 3' labelled with the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA). Primers and probes were purchased from Applied Biosystems, UK (Table 1).

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TaqMan[®] assay conditions

Each real-time PCR reaction contained 1 × TaqMan[™] Buffer A, 25 mM MgCl₂ solution, 0.625 units AmpliTaq Gold[®] DNA Polymerase (Applied Biosystems) and 0.5 μM of each deoxynucleotide triphosphate (Sigma) in 25 μL. Primers were used at 900 nM and probes at 200 nM. DNA template was diluted in water and 5 μL of either DNA or water, as a negative control, was added per reaction. Reactions were assembled in Axygen thin walled 96 well plates with optical caps and run on ABI Prism 7700 or 7900 sequence detection systems with the following thermal cycling protocol: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

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Results and Discussion

The effect of amplicon length on amplification rates

It is known that food heat and pressure processing can result in DNA fragmentation which can, in turn, have implications on the sensitivity of DNA based analyses. We designed real-time PCR primers and probe systems that would enable us to determine the degree of DNA fragmentation after processing, by using amplicons of different lengths. The reverse primer remained constant (anchored in position) and was paired with a series of 8 alternative forward primers, giving amplicons of increasing size (81, 116, 240, 317, 351, 427, 470 and 565 base pairs) (Figure 1).

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[Figure 1 to go about here]

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4 Primers were identified as close as possible to target sizes, for example amplicon
5 length of 120 base pairs was not possible for this region of turkey sequence and
6 therefore a primer was designed which would give an amplicon as close to 120 base
7 pairs as possible, therefore the design of the primers was largely dependant upon the
8 sequence. Additionally, the Primer Express design parameters were relaxed for
9 amplicons above 150 base pairs to allow the design of primers giving amplicons
10 larger than 150 base pairs: the usual upper limit for real-time amplicons (Table 1).

11 [Table 1 to go about here]

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19 These primer sets were then used to investigate the effect of amplicon length on
20 amplification rates using raw template DNA (Figure 2). An increase in C_T value
21 (threshold cycle), indicating a reduction in amplification rate was observed with
22 increasing amplicon size for the turkey primer series. Amplicons up to 240 base pairs
23 had similar amplification rates, whereas for amplicon sizes above 240 base pairs the
24 increases in C_T values were more marked, particularly between amplicons of 427 and
25 565 base pairs, where there was an increase of 5 C_T .

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31 The results from these experiments are in accordance with the technical information
32 supplied by Applied Biosystems. The reactions of *Taq* polymerase are catalysed by
33 magnesium and the enzyme has a large tolerance for magnesium concentration with
34 small amplicons. Amplicon sizes below 150 base pairs are recommended since the
35 magnesium concentration can remain constant, however the data presented here
36 suggests that amplicons above 150 base pairs can also support efficient amplification,
37 since there was a rise of only 1.5 C_T when the amplicon size was increased to over
38 300 bp. These data support the view that for abundant template molecules amplicon
39 size should be kept below 150 base pairs, however for sequences which prove
40 difficult for primer and probe design, amplicon sizes above 150 base pairs may also
41 support efficient amplification.

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49 Figure 2 to go about here,

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53 *Assessment of the degree of DNA fragmentation during processing*

Deleted: Primers were identified to give amplicons of target sizes (70, 120, 350 and 500 base pairs) although the actual lengths of the amplicons was dependant upon the sequence. Design parameters of Primer Express, the package used to design TaqMan primer and probe systems, were relaxed for amplicons above 150 base pairs: the usual upper limit for real-time amplicons

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Deleted: These primer sets were then used to investigate the effect of amplicon length on amplification efficiency using raw template DNA (Figure 2). An increase in threshold cycle (C_T) value, indicating a reduction in amplification efficiency was observed with increasing amplicon size for all species. For the chicken and cow primer series, amplicons up to 353 base pairs (primer sets 1, 2, & 3) had similar efficiencies, whereas amplicon sizes above 353 base pairs (primer set 4) were found to have statistically significant differences and the increase in C_T values became marked. A similar pattern was observed in the data for the turkey and pig primer series where amplicons of approximately 70 and 120 base pairs (primer sets 1 & 2) had similar efficiencies, whereas amplicons with sizes of 312 base pairs for turkey and 350 base pairs for pig, and above (primer sets 3 & 4) were found to have statistically significant increases in C_T value. ¶

¶ [Figure 2 to go about here]¶

¶ The reactions of *Taq* polymerase are catalysed by magnesium, amplicon sizes below 150 base pairs are recommended since the magnesium concentration should remain constant for small amplicons. The data presented here suggests that larger amplicons may support efficient amplification. Any reduction in efficiency is unlikely to be critical as the sensitivity of these types of assays is rarely a limiting factor and may be negated or reduced by the optimisation of magnesium concentration. ¶ There were two broad trends in the data from the four primer series: the cow and chicken series appeared to amplify with similar efficiencies as did the turkey and pig series. We used a primer series displaying each trend to examine the effect of diluting the target species in a meat background. Mixtures of cow in pig or turkey in pig were prepared at 95, 90, 80, 50, 20, 10 and 5% [w/w] cow or turkey. The DNA was extracted from duplicate samples and then analysed using the real-time PCR primer series for cow and turkey (Figure 3, 5%, 50% and 95% data shown). It was found that the trends in amplification efficiency, decreasing with amplicon size, were again displayed in these data sets. For cow, amplification efficiencies were similar for amplicons of 62, 109 and 353 bp (primer set 1, 2, & 3), whereas turkey amplicons of 76 and 111 base pairs (primer set 1 & 2) had similar

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To investigate the effect of heat and pressure processing on the availability of template molecules, we used the turkey primer series to amplify DNA which had been extracted from turkey muscle which had been subjected to the following 6 different cooking conditions: light boiling, heavy boiling, light baking, heavy baking, canning and autoclaving. The DNA was extracted from duplicate samples, the DNA concentration adjusted to 17ng/ul and then 85ng/reaction analysed using the real-time PCR primer series for turkey (Figure 3).

[Figure 3 to go about here]

We found that, in general terms, there was no difference in the amplification rates of DNA extracted from samples which had been boiled or baked, in comparison to DNA from raw samples for amplicons of 81, 116, 351 or 565 base pairs. This indicated that the fragmentation of the DNA had not affected amplification for amplicons up to 565 base pairs since the amplification rates were roughly similar to those of raw samples, regardless of the boiling or baking conditions. This also indicated that amplicons of 351 and 565 base pairs are able to efficiently amplify template DNA extracted from boiled or baked samples.

There was a statistically significant increase in the C_T value returned for the canned and autoclaved samples for all sizes of amplicons. This difference became more pronounced with increasing amplicon size, where amplification of DNA extracted from the autoclaved sample returned a value 1.41 C_T greater than amplification of the DNA extracted from the raw sample with the 81 base pair primer set compared to an increase of 10 C_T for the 351 base pair primer set and a complete failure for the 565 base pair primer set. A similar trend was observed for the canned samples, where amplification rates decreased significantly with increasing amplicon length.

Fragmentation of DNA was only observed when the meats were canned or autoclaved, with little or no fragmentation observed when the meats were cooked in the absence of pressure. The effect of DNA fragmentation on PCR based systems is two fold. Firstly, when DNA fragments, the available template molecules are reduced when a template molecule is cleaved. Secondly, small DNA fragments are not

Deleted: The current method of choice for the identification of cooked or canned meat or fish usually involves the use of PCR. The degree of processing of food, and therefore the degree of DNA fragmentation, has a profound effect on the accuracy and robustness of the PCR. There is little information on the effect of heat and pressure on DNA fragmentation. It has been determined that following autoclaving the average fragment size of pig DNA was reduced to 300 bp (Meyer Candrian and Luthy 1993), however the exact effects of processes, normally encountered in the production of commercial products, on the detection of DNA are unknown. ¶

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Deleted: trends we had previously observed were again repeated for these data sets. The amplification efficiencies of the cow primer series were similar across the raw, boiled and roasted samples, however the efficiencies were lower for the canned samples. Similar data was observed for the turkey primer series where it was only in the canned samples where the effects of processing could be seen.

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extracted with the same efficiency as larger template molecules by some DNA extraction systems, particularly for extraction techniques which use DNA precipitation, since small fragments of DNA do not precipitate as easily as large fragments.

In this study the effect of DNA extractability from heat and pressure processed matrices on amplification rates may have contributed to the increased C_T values observed in the canned and autoclaved samples although this is an unlikely cause for the increased C_T value since there was little effect of canning and autoclaving on the amplification rate of the 81 base pair amplicon. Had DNA extractability from the canned and autoclaved samples been a major contributor to the increased C_T value returned for amplicons of 351 and 565 base pairs, we would have also observed a significant increase in the C_T values returned for amplicons of 81 and 116 base pairs. These data indicated that canning and autoclaving affected the fragmentation of the DNA, rather than the DNA extractability.

To further illustrate the implication of amplicon size on the detection of meats in a mixture we used the turkey primer series to analyse a sample of poultry meal, containing approximately 15% turkey. Meal of this type is heated to 100°C for 60 minutes, in the absence of pressure, where the final temperature reached is 120 °C resulting in fragmented DNA. Since only a proportion of the sample was derived from turkey the raw data is to show the effect of amplicon size on rates of amplification and is not for direct comparison. Nevertheless, DNA fragmentation was evident in this sample since the C_T value differential between the raw and processed samples increased with increasing amplicon size indicating fragmentation of target DNA (Figure 4). However it should be noted that an amplicon of 351 base pairs was still detectable with a C_T value of 33.87 in this processed sample.

Figure 4 to go about here

Conclusions

Overall this study showed that the degree of DNA fragmentation correlated with the degree of heat and pressure processing and that this could be assessed using multi-

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[Figure 5 to go about here]¶

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2 primer assays. DNA fragmentation was minimal for samples which had been baked
3 or boiled and turkey DNA extracted from autoclaved samples was easily detectable
4 even using amplification systems spanning 351 base pairs. This study proved that
5 amplification rates were not compromised for amplicons of more than 150 base pairs
6 and that in future, the design of real-time PCR assays could afford greater focus on
7 achieving specificity using a less stringent approach to limiting amplicon size.
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Figure 1. Strategy for determining the degree of DNA fragmentation following heat and pressure processing.

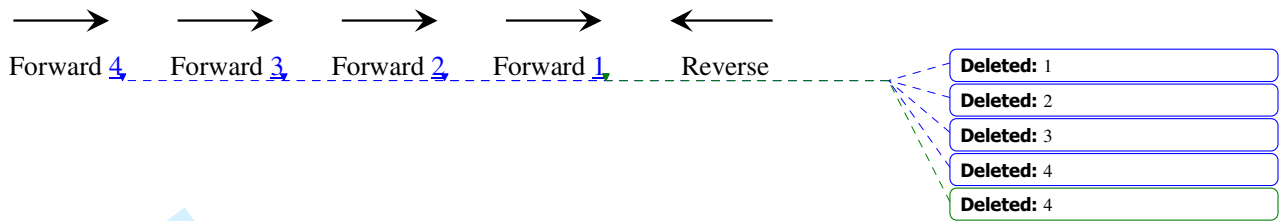
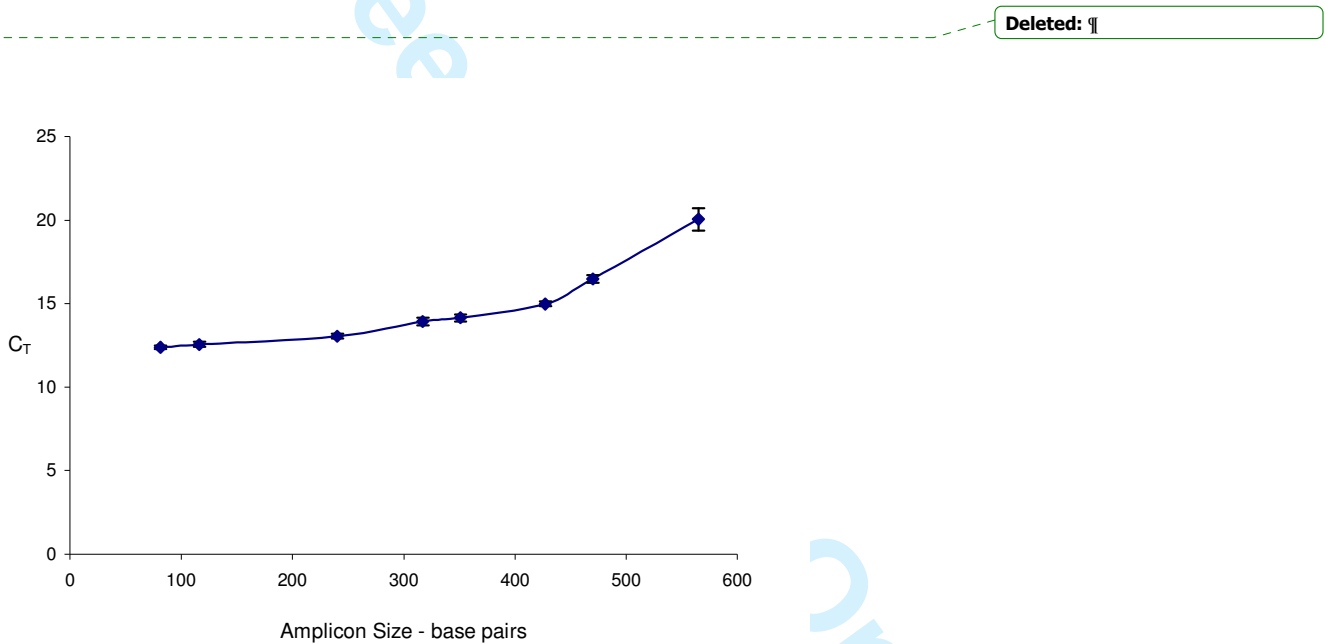


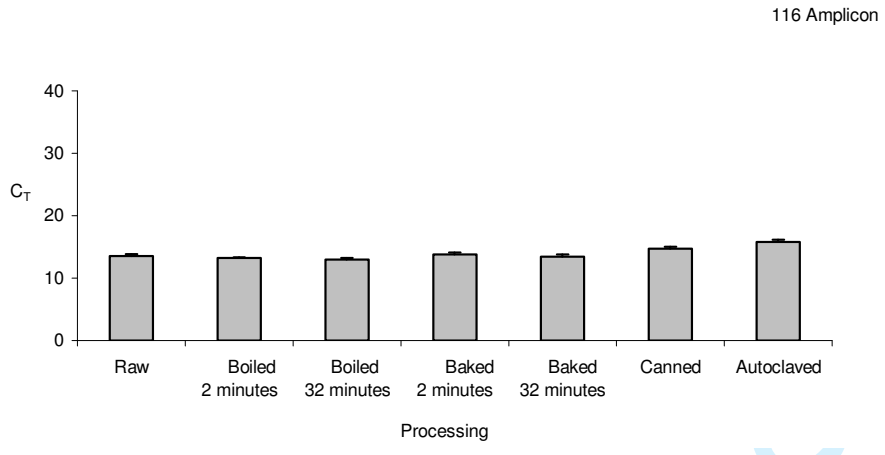
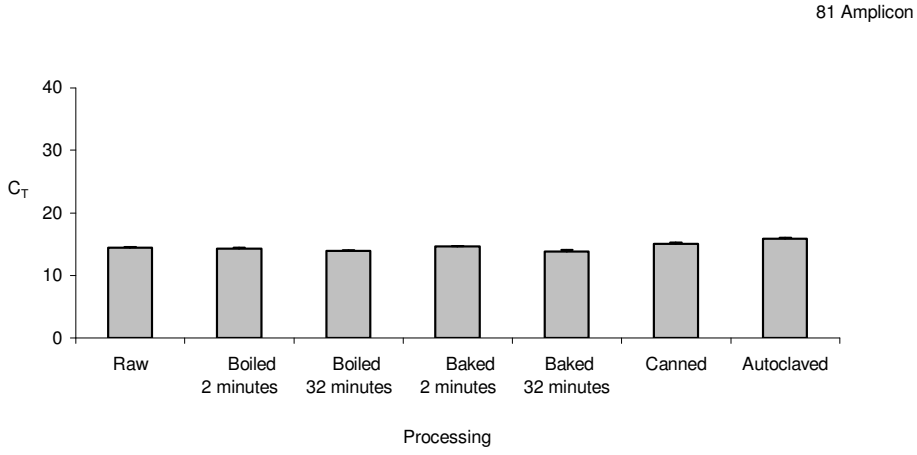
Figure 2. Effect of amplicon size on amplification rates of turkey primer series using raw turkey DNA as template average and standard deviations shown



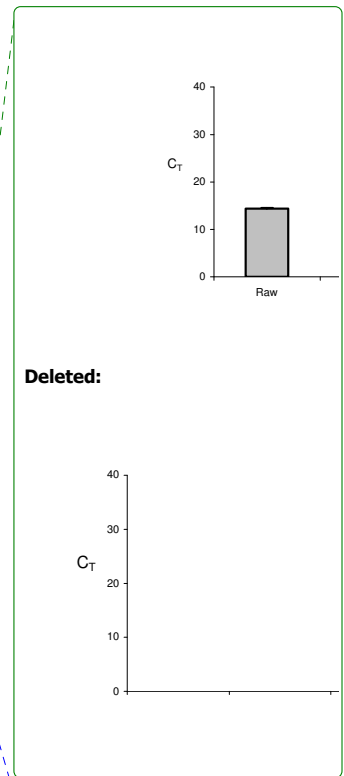
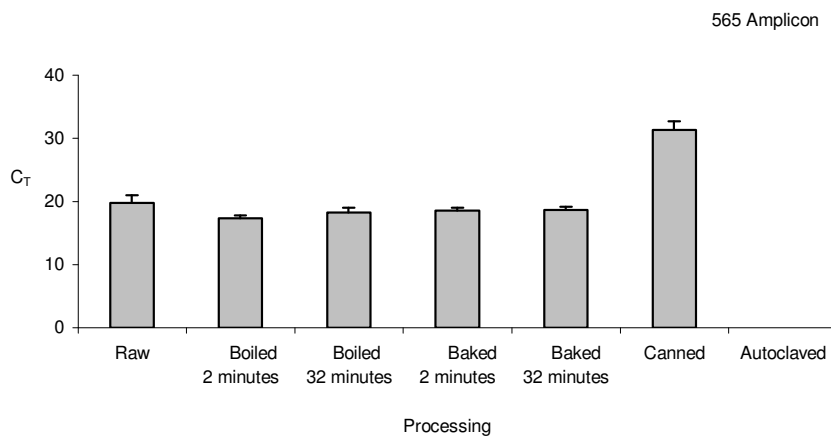
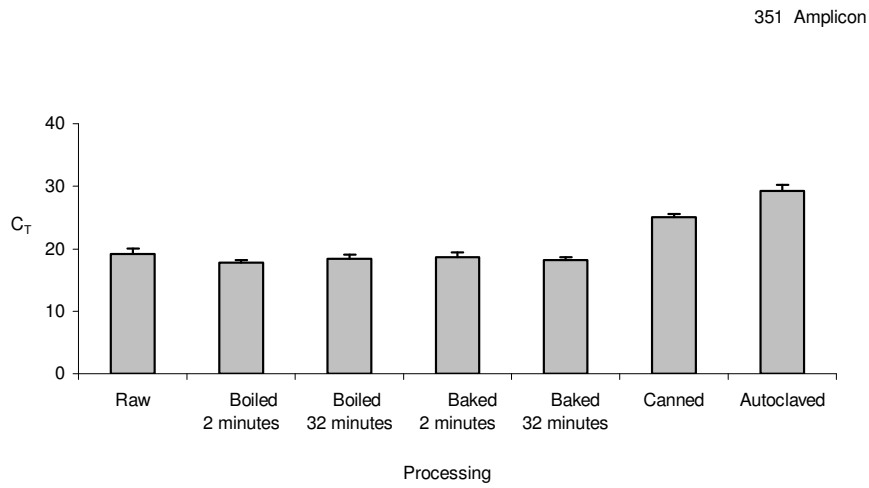
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Figure 3 The effect of heat and/or pressure processing on the amplification rates of turkey specific assays giving amplicons of 81, 116 351 and 565 base pairs, mean and standard deviations shown

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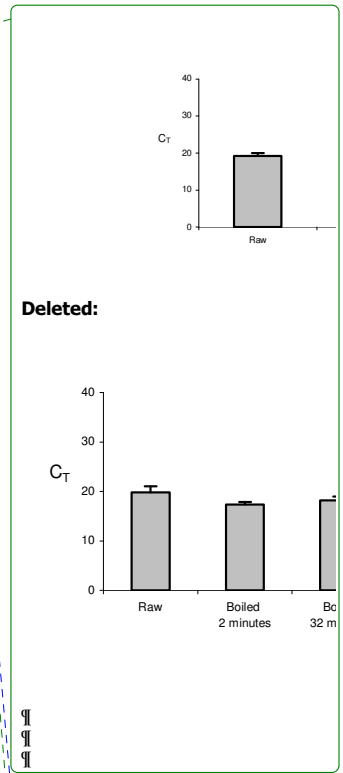
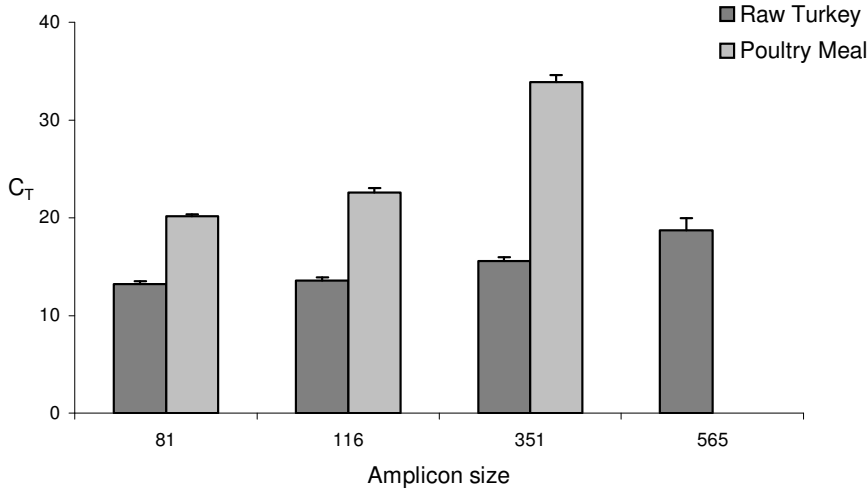
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Figure 4. The detection of turkey in poultry meal using primer sets to give amplicons of 81, 116, 351 and 565 base pairs, average and standard deviations shown.



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81 bp Amplicon		Ct
Raw A		13.0
		13.0
		13.0
Raw B		12.0
		12.0
		13.0
56A1		20.0
		20.0
		20.0
56A2		20.0
		20.0
		20.0
56B1		20.0
		20.0
		20.0

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Table 1. Sequence and amplicon length of primer and probe sets used in this study.

Species	Oligonucleotide	Sequence (5'-3')	Amplicon length (bp)
Turkey	Forward 1	AAA CCA GAG TGA TAC TTT CTA TTT	81
	Forward 2	CCC CAG CAA ATC CAC TAG T	116
	Forward 3	ACA AAA TCC CAT TCC ACC CC	240
	Forward 4	CAG GAA TTA CAA TCA TCC AC	317
	Forward 5	TCG CCC TCC ACT TCC TCC	351
	Forward 6	AAC CCT AGT AGA ATG GGC CTG AG	427
	Forward 7	CAT TCT GAG GGG CTA CC	470
	Forward 8	CTA TAT AAA GAA ACC TGA AATA CAG GAG TAG TCT	565
	Reverse	AGG CTA GGA CAC CTC CA	-
Probe	FAM - ACG CAA TCC TAC GCT CAA TCC CAA ACA- TAMRA	-	

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These primer sets were then used to investigate the effect of amplicon length on amplification efficiency using raw template DNA (Figure 2). An increase in threshold cycle (C_T) value, indicating a reduction in amplification efficiency was observed with increasing amplicon size for all species. For the chicken and cow primer series, amplicons up to 353 base pairs (primer sets 1, 2, & 3) had similar efficiencies, whereas amplicon sizes above 353 base pairs (primer set 4) were found to have statistically significant differences and the increase in C_T values became marked. A similar pattern was observed in the data for the turkey and pig primer series where amplicons of approximately 70 and 120 base pairs (primer sets 1 & 2) had similar efficiencies, whereas amplicons with sizes of 312 base pairs for turkey and 350 base pairs for pig, and above (primer sets 3 & 4) were found to have statistically significant increases in C_T value.

[Figure 2 to go about here]

The reactions of *Taq* polymerase are catalysed by magnesium, amplicon sizes below 150 base pairs are recommended since the magnesium concentration should remain constant for small amplicons. The data presented here suggests that larger amplicons may support efficient amplification. Any reduction in efficiency is unlikely to be critical as the sensitivity of these types of assays is rarely a limiting factor and may be negated or reduced by the optimisation of magnesium concentration.

There were two broad trends in the data from the four primer series: the cow and chicken series appeared to amplify with similar efficiencies as did the turkey and pig series. We used a primer series displaying each trend to examine the effect of diluting the target species in a meat background. Mixtures of cow in pig or turkey in pig were prepared at 95, 90, 80, 50, 20, 10 and 5% [w/w] cow or turkey. The DNA was extracted from duplicate samples and then analysed using the real-time PCR primer series for cow and turkey (Figure 3, 5%, 50% and 95% data shown). It was found that the trends in amplification efficiency, decreasing with amplicon size, were again displayed in these data sets. For cow, amplification efficiencies were similar for amplicons of 62, 109 and 353 bp (primer set 1, 2, & 3), whereas turkey amplicons of 76 and 111 base pairs (primer set 1 & 2) had similar amplification efficiencies and larger amplicons had reduced

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3 efficiencies, regardless of the percentage of the target meat species. It appeared that the
4 presence of non-target DNA template had no effect on the amplification efficiencies of
5 both primer series and that at the 5% level the detection of raw template DNA was
6 achieved equally well by amplification systems using amplicons up to 350 and 111 base
7 pairs for cow and turkey respectively.
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14 *[Figure 3 to go about here]*
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17 These data indicate that the fragmentation of DNA for the raw, boiled and roasted
18 samples was similar and that there was significantly more fragmentation following
19 canning, which was evident, even using the smallest primer sets, with a relatively small
20 amplicon size of below 80 base pairs for both turkey and cow.
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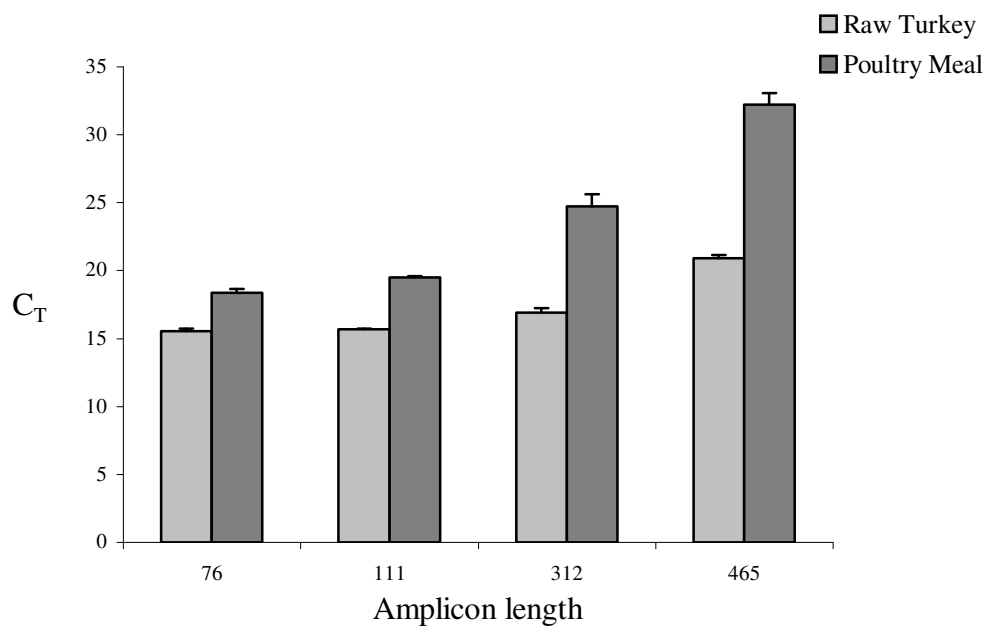
25 The mixtures analysed during the course of this study were all prepared in a pig meat
26 background and
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31 The inclusion of canned samples was instrumental in the assessment of processing
32 conditions since we have previously found that autoclaving meat does not mimic the
33 canning process and has little effect on the fragmentation of DNA in comparison.
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