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## Introduction

The need for reliable and sensitive methods for meat species identification encompasses many issues including the fraudulent substitution of cheaper/lower quality meats in place of more expensive ones [1]. Besides representing a commercial fraud, incorrect labeling is also a problem for religious issues, since pork meat is forbidden in some religions. Following the European labeling regulations, meat products should be accurately labeled regarding their species content. From several analytical methodologies developed to accomplish meat product authentication, DNA analysis coupled with polymerase chain reaction (PCR) presents a fast, sensitive and highly specific alternative to other methods [2]. The aim of this work was to develop PCR techniques able to identify and quantify different ingredients (pork, poultry and beef) in highly processed meat product, such as Frankfurt sausages.

## Methodology



Food samples and standards (binary mixtures with known percentages of pork/poultry) were extracted by two methodologies: the CTAB method, based on liquid-liquid extraction, and the Wizard method, based on silica solid-phase extraction as described by Lipp et al. (1999) [3]. Yield and purity of extracts were assessed by agarose gel electrophoresis and by spectrophotometry.

All PCR amplifications were performed in a iCYCLER BIO-RAD thermocycler using AmpliTaq Gold<sup>®</sup> DNA polymerase (Applied Biosystems). The oligonucleotide primers used are presented in Table 1. The PCR amplification conditions and components used, are presented in Tables 2 and 3. SYBR Green I dye (Applied Biosystems) was used for real-time PCR assays.

Table 1 – Oligonucleotide primers

Species	Primers	Sequence 5'-3'	Amplicon (bp)	Reference
<b>Qualitative PCR</b>				
Pork	SUS-F	CTA CAT AAG AAT ATC CAC CAC A	290	Dalmasso et al 2004
	SUS-R	ACA TTG TGG GAT CTT CTA GGT		
Poultry	GAL-F	TGA GAA CTA CGA GCA CAA C	183	Dalmasso et al 2004
	GAL-R	GGG CTA TTG AGC TCA CTG TT		
Cow	916	GTA CTA CTA GCA ACA GCT TA	256	Botero et al 2003
	1171	GCT TGA TTC TCT TGG TGT AGA G		
<b>Quantitative PCR</b>				
Pork	Pork-F	ATG AAA CAT TGG AGT CCT ACT TT TAC C	140	Dooly et al 2004
	Pork-R	CTA CGA GGT CTG TTC CGA TAT AAG G		
Poultry	The same as in qualitative PCR			

Table 2 – PCR Amplification conditions

Target Primers	Pork Sus-F/Sus-R	Poultry Gal-F/Gal-R	Cow 619/1171	Real-Time PCR
<b>Steps</b>	<b>Temp.</b>	<b>Time</b>	<b>Temp.</b>	<b>Time</b>
<b>Denaturation</b>	94°C	5 min	94°C	5 min
<b>Amplification</b>	94°C	30 s	94°C	30 s
	60°C	1 min	60°C	1 min
	72°C	1 min	72°C	1 min
<b>Nº of cycles</b>	35	38	35	45
<b>Final extension</b>	72°C	5 min	72°C	5 min
				*

\* Real time-data collection after each cycle; Increase temperature of 0.5°C (from 65 to 94°C) and collection of data for melting curve; conditions applied for Pork, Poultry and Soybean PCR-RT tests.

Table 3 – Qualitative PCR components

Component	Reaction volume (µL)		
	Pork	Poultry	Beef
Ultrapure water	13.8	13.55	14.8
Buffer (10 x)	2.5	2.5	2.5
MgCl <sub>2</sub> (25 mM)	2.0	2.25	2.5
dNTPs (2.5 mM each)	2.0	2.0	2.0
Primers (10mM each)	1.25	1.25	0.5
Taq Polymerase (5U/µL)	0.2	0.2	0.2
DNA extract	2.0	2.0	2.0
<b>Total reaction volume</b>	<b>25</b>	<b>25</b>	<b>25</b>

## Results

### Qualitative PCR

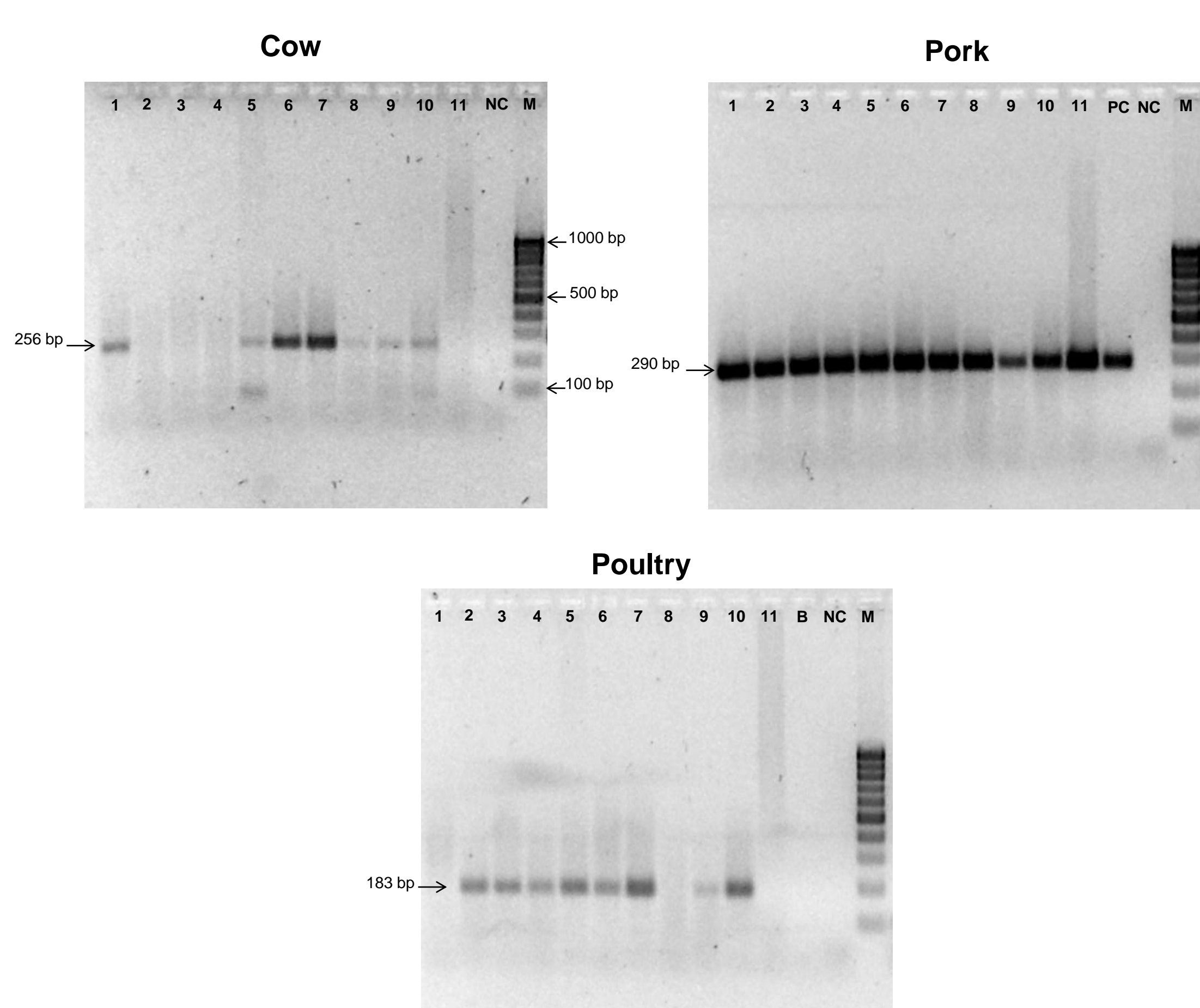


Figure 1 – Agarose gel electrophoresis for PCR products of specific detection of pork, poultry, beef and soybean. M: 100 bp ladder; Samples: 1-11, B: blank, CN: negative control, CP: positive control.

### Real-time PCR

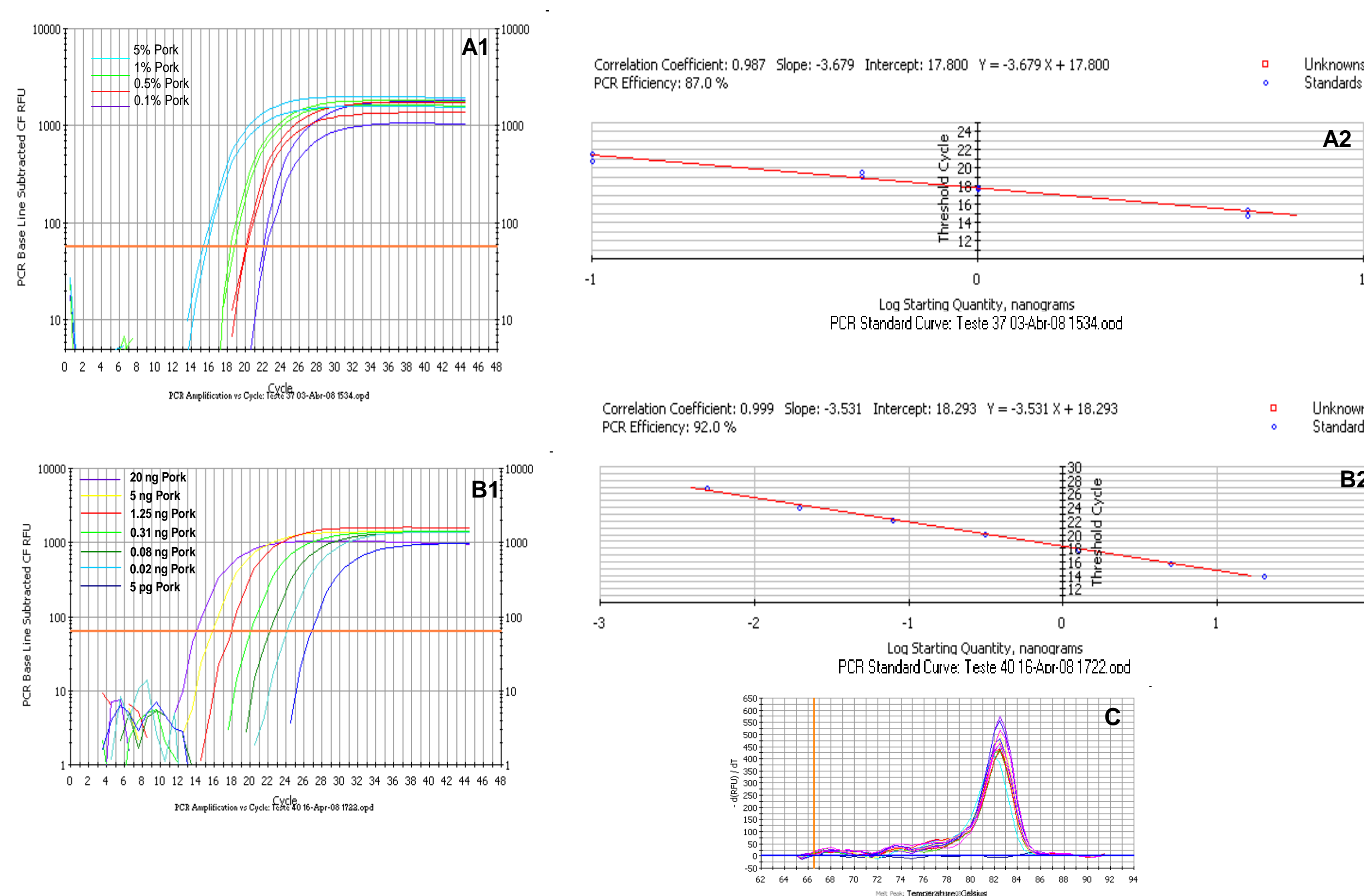


Figure 2 – A1, B1: Fluorescence signal with SYBR Green I dye vs. Cycle number (A: binary mixtures of pork/soybean; C: 100% pork sample); A2, B2, C: Melting curves for the 140 bp fragment of pork species.

Table 4 – Agarose gel electrophoresis for PCR products of specific detection of pork, poultry, beef and soybean.

Samples	Pork		Poultry		Milk protein	
	Labelled	Detected	Labelled	Detected	Labelled	Detected
1	✓	✓	✓	✗	X	✓
2	✓	✓	✓	✓	✓	✗
3	✓	✓	✓	✓	X	X
4	✓	✓	✓	✓	✓	✗
5	✓	✓	✓	✓	✓	✓
6	✓	✓	X	✓	✓	✓
7	✓	✓	✓	✓	✓	✓
8	✓	✓	X	X	✓	✓
9	X	✓	✓	✓	✓	✓
10	X	✓	✓	✓	✓	✓
11	✓	✓	X	X	X	X

## Conclusion

- ✓ The developed techniques allowed the detection of the tested species (pork, poultry and cow). The methodologies were successfully applied in commercial samples of frankfurters.
- ✓ Qualitative PCR assays showed the presence of undeclared pork species in 2 samples, undeclared poultry species in 1 sample and undeclared cow species in 1 sample (Table 4). In one sample, the declared poultry species was not detected. The same happened in 2 samples labelled as containing milk protein.
- ✓ Real-time PCR assays showed high sensibility (0.1%) for pork (Fig. 2) and poultry (data not shown)
- ✓ The high real-time PCR efficiency and high correlation coefficients obtained (Fig. A2, B2) suggest that this techniques proved to be adequate for future quantitative assays