The impact of processing technologies and isolation procedures on the quality of animal DNA in food and feed products

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Abstract

The aim of this study was to find suitable methods for the homogenisation and isolation of nucleic acids from samples of raw and thermally or mechanically treated muscle tissue and various types of meat products and pet foods. The effect of various processing methods (high temperature, pressure, mechanical processing, and addition of other raw materials) on DNA from animal tissues (chicken, pork, beef) in products manufactured in technology workshops was investigated. Subsequently, a number of extraction techniques for DNA isolation from model meat products were tested. DNA quality and quantity were assessed by absorbance measurements and determination of the ratio of absorbance using a spectrophotometer. Gel electrophoresis was performed in order to determine the degree of DNA fragmentation induced by technological processing. The amplification ability was tested by PCR analysis. Finally, the financial and manual demands of extraction kits were compared.

DNA isolation, food authenticity, PCR analysis

Introduction

Verification of food authenticity is a fundamental issue in food safety. The legislation of the Czech Republic is based on the valid European Union legislation in the area of food safety. Verification of species origin in foods and feeds is important primarily in supporting the application of the law and for the protection of consumers in terms of their health and economic and religious interests. Food labelling does not always guarantee total surety, for which reason analytical methods must be used for species identification. Such analysis focuses, first and foremost, on the detection of proteins or DNA molecules extracted from the tissue. In view of the denaturation of proteins caused by thermal treatment or the conserving process (high temperature combined with high pressure) (Mackie et al. 1999), DNA is a more suitable molecular marker for species determination as it is more resistant to thermal processing. In fact, DNA is degraded during the thermal process into smaller fragments, though these are still detectable. DNA is, what's more, largely independent of the source of the tissue or damage to the sample (Bossier et al. 1999; Lockley and Bardsley 2000). The crucial step is the extraction of a sufficiently large quantity of high-quality DNA from heterogeneous food matrices. In certain cases, preparatory treatment of the sample is required before DNA extraction (e.g. the removal of fat from the tissue). In view of the fact that raw muscle is subjected to varying processing conditions during the production process (high temperature, high pressure, addition of certain ingredients, etc.) that significantly influence DNA quality (Buntjer et al. 1999; Musto 2011; Camma et al. 2012), DNA isolation procedures must be optimised on an individual basis for each type of food product. In addition, various ingredients and other chemical compounds present in food matrices (polysaccharides, proteins, collagen, polyphenols, fulvic acids and lipids) might not be eliminated completely during DNA extraction and may influence the integrity of the DNA from the viewpoint of PCR inhibition. Inhibitor compounds may interfere with PCR by reducing or even completely inhibiting the activity of DNA

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polymerase (Di Pinto et al. 2007). The aim of this study was to determine the degree to which DNA was affected by technological processes used in the food industry (mechanical, thermal, chemical and enzymatic treatment) in samples we prepared ourselves from the muscle tissue of pigs, chickens and cattle, and how the subsequent preparation of samples and the DNA extraction procedure may affect its qualitative and quantitative parameters.

Materials and Methods

Sample preparation

Authentic fresh pork meat (*Sus scrofa domesticus*), chicken meat *(Gallus gallus*) and beef meat *(Bos taurus*) was purchased on the local retail network or provided by a local meat plant. Formulations of selected types of meat products were optimised for the production of samples. The model samples were comprised of samples of the individual species, meat mixes and products containing a defined percentage of the studied species muscle (chicken, pork or beef). Lean pork muscle and fatty pork muscle was chosen in view of the fact that the natural heterogeneity of the tissue composition may influence the effectiveness of DNA extraction. The basic series was comprised of samples of lean pork or fatty pork muscle, chicken muscle and mixes of samples defined by ratios that were further subjected to a thermal process (mincing followed by thermal processing at 70 °C / 10 minutes), a boiling process (mincing followed by thermal processing at 100 °C / 10 minutes) and conserving (cutting and mixing followed by thermal processing at 121.1 ° C / 10 minutes). Another set of model samples was comprised of completed meat products: the heat-treated meat product Vienna sausage, a non-heat-treated meat product (Teewurst) and a durable non-heat-treated fermented meat product of the "Poličan" salami type. These meat products were made in accordance with the product formulations of the manufacturers (2 different percentages of the individual meat ingredients) at the technological research laboratories at the University of Veterinary and Pharmaceutical Sciences in Brno (Czech Republic). Two samples of commercial pet foods with a defined composition – granulated and canned pet foods – were also tested. Table 1 describes the specific model samples.

DNA isolation

DNA was isolated in duplicate with the use of eight commercially available extraction kits. Six commercial sets were based on the affinity of DNA to bond to the silica membrane column - DNeasy Blood and Tissue Kit (Qiagen), DNeasy mericon Food Kit (Qiagen), Food DNA Isolation Kit (Norgen Biotek), UltraPrep Genomic DNA Food Mini Prep Kit (AHN-Bio), High Pure PCR Template Preparation Kit (Roche) and NucleoSpin Food (Macherey-Nagel). One commercial set based on magnetic separation was used - Chemagic DNA Tissue10 (PerkinElmer) and the final kit was based on phenol-chloroform extraction. The extraction procedures were performed according to the protocols supplied by the manufacturers. Sample weights ranged from 10 mg to 200 mg depending on the kit in question. Proteolysis was performed overnight in all extraction protocols.

DNA quantification and purity determination

The quality of the extracted DNA was compared by measuring its concentration and purity with a UV spectrophotometer (NanoDrop™ 1000, Thermo Scientific). DNA extracts were quantified by measuring absorbance at 260 nm (A260). DNA purity was determined by calculating A260 / A280 ratios. Samples giving values within a range of 1.7 – 2.0 calculated on the basis of the A260 / A280 ratio are considered pure samples without undesirable substances (e.g. remnants of proteins or other contaminants). Measurement was performed at room temperature with sufficient vortexing of all samples.

DNA fragmentation and PCR amplification

The basic separation method which enables the identification or purification of nucleic acids is electrophoresis (Zvarová et al. 2012), and gel electrophoresis was performed to determine DNA fragmentation for the purpose of checking the degree of DNA integrity which is generally impaired in processed samples. Primers targeted at the amplification of fragments of defined sizes (beef 274 bp, pork 398 bp, chicken 227 bp) were used to check the suitability of the extracted DNA for subsequent PCR analysis. Primers were used according to Matsunaga et al. (1999). Forward primers are the same for all three types, while the reverse primers for chicken pork and for beef are (5´-AAGATACAGATGAAAGAAGAATGAGGCG- 3´), (5´- GCTGATAGTAGATTTGTGATGACCGTA- 3´) and (5´-CTAGAAAAGTGTAAGA- 3´), respectively. The PCR protocol was performed in the following steps: denaturation at 94 ºC for 30 s, annealing at 60 ºC for 30 s and extension at 72 ºC for 30 s. PCR products were further subjected to electrophoresis for 30 min at 100 V.

Results and Discussion

DNA is exposed to many factors in processed products (the action of heat, physical or chemical treatment) which may lead to the fragmentation of DNA molecules. The quality and yield of extracted DNA are critical factors in the preparation of DNA for further

Table 1. Model samples

Sample	Chicken	Lean pork	Fatty pork [%]	Pork lard	Beef	Degree of processing	Other additives
1	0	100	0	0	0	raw	-
2	0	100	0	0	0	70 ºC / 10 min	-
3	0	100	0	0	0	100 ºC / 10 min	-
4	0	100	0	0	0	120 ºC / 10 min	-
5	90	10	0	0	0	-	-
6	90	10	0	0	0	70 ºC / 10 min	-
7	90	10	0	0	0	100 ºC / 10 min	-
8	90	10	0	0	0	120 ºC / 10 min	-
9	0	0	100	0	0	-	-
10	0	0	100	0	0	70 ºC / 10 min	-
11	0	0	100	0	0	100 ºC/10 min	-
12	0	0	100	0	0	120 ºC / 10 min	-
13	90	0	10	0	0	-	-
14	90	0	10	0	0	70 ºC / 10 min	-
15	90	0	10	0	0	100 ºC / 10 min	-
16	90	0	10	0	0	120 ºC / 10 min	-
17	12	50	0	10	28	Vienna sausage	
18	31	31	0	10	28	Vienna sausage	
19	50	12	0	10	28	Vienna sausage	
20	0	20	0	80	0	Teewurst 1	
21	0	40	0	60	0	Teewurst 2	
22	0	38	0	33	29	ferment 1	
23	8	32	0	30	30	ferment 2	
24	+	+	?	?	-	granulated dog food	*
25	43	35	?	?	20	canned cat food	vitamins, minerals

*Dehydrated chicken meat, maize gluten, dehydrated pork meat, pea fibre, oats, hydrolysed animal proteins, spelt, dehydrated fish, animal fat, dehydrated egg, fish fat, mannan-oligosaccharides, psyllium, sodium chloride, calcium sulphate, glucosamine, marigold extract (source of lutein)

PCR analysis. A number of factors have to be taken into consideration for us to select the optimal extraction procedure. The DNA should contain the smallest possible amount of contaminants (proteins, polyphenols, polysaccharides) and any other PCR inhibitors. The DNA concentration and purity were determined by spectrophotometric measurement based on DNA absorbance and A260 / A280 ratios. DNA is considered satisfactorily pure when the A260 / A280 ratios are within a range of 1.7 – 2.0. Contamination of DNA with proteins generally reduces the A260 / A280 ratio to values lower than 1.7. Residual impurities from DNA extraction, such as phenols or ethanol, also reduce the A260 / A280 ratio. In this context, residual chemical contamination caused by extraction of nucleic acids may lead to overestimation of the concentration of nucleic acid. The suitability of DNA for PCR amplification was assessed by PCR according to Matsunaga et al. (1999).

Average values of DNA concentration [$ng \cdot \mu l^{-1}$] for specific extraction techniques are depicted in Fig. 1. The concentration values are based on the linear dependence of nucleic acid and absorbance measurements in the UV spectrum at 260 nm. The possibility of false results exists as all substances in the solution with absorbance at 260 nm are measured. If, for example, the DNA is contaminated, a DNA concentration higher than the true concentration may be measured. This may be avoided by the use of an appropriate washing

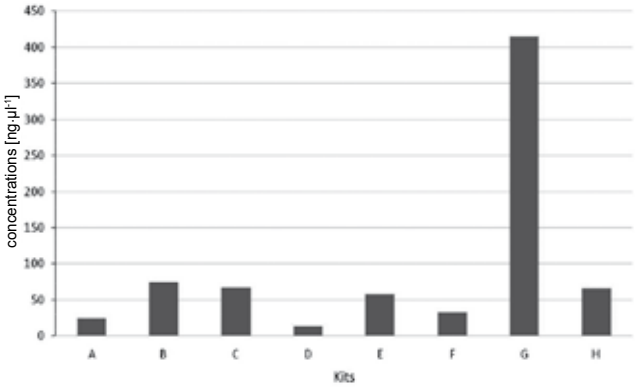

Fig. 1. Average values of DNA concentrations for individual kits (A–H)
*A – DNeasy Blood & Tissue Kit (Qiagen), B – DNeasy mericon Food Kit (Qiagen), C – Chemagic DNA Tissue 10 Kit (PerkinElmer), D – Food DNA Isolation Kit (Norgen Biotek), E – UltraPrep Genomic DNA Food Mini Prep Kit (AHN-Bio), F – High Pure PCR Template Preparation Kit (Roche), G – Phenol-chloroform extraction, H – NucleoSpin Food (Macherey-Nagel)

step during DNA extraction. The highest DNA concentrations were observed with kit G (phenol-chloroform extraction), though these results could be misleading due to the process used during DNA extraction (chemical agents with a less thorough purification step) and this effect may have been caused by residual chemical contaminants. The lowest DNA yield was detected with the use of kit D. Although the DNA yields differed significantly depending on the type of extraction method, the purity of almost all the extracts obtained fell within an A260 / A280 ratio range of 1.7 – 2.0 (Table 2), with the exception of kits C, E and F. We obtained information about the approximate purity of the isolated DNA, but not about the potential fragmentation that occurred during the isolation procedure.

Table 2. Range of "A" values for individual kits

Extraction kit*	"A" value		
	< 1.7	1.7 – 2.0	> 2
A	2	48	0
B	3	46	1
C	39	11	0
D	2	24	24
E	45	5	0
F	49	1	0
G	1	45	4
H	20	22	8

*A – DNeasy Blood & Tissue Kit (Qiagen), B – DNeasy mericon Food Kit (Qiagen), C – Chemagic DNA Tissue 10 Kit (PerkinElmer), D – Food DNA Isolation Kit (Norgen Biotek), E – UltraPrep Genomic DNA Food Mini Prep Kit (AHN-Bio), F – High Pure PCR Template Preparation Kit (Roche), G – Phenol-chloroform extraction, H – NucleoSpin Food (Macherey-Nagel)

Integrity is another qualitative parameter in DNA isolation. Isolated DNA is always degraded (the degree of fragmentation may, however, differ), so the result of electrophoresis is not a sharp band, but a considerably diffuse band (a smear). When comparing various isolation methods, the better method is the one that results in the more compact band and

larger molecular weight. Regardless of the method of extraction, a considerable degree of DNA degradation was observed in all samples. Some extraction methods lead to greater DNA fragmentation (a blurred band without a clear maximum) depending on the specific sample, while some have low yield (a low yield was observed in almost all samples with the use of methods D, E and G). A low yield was found with almost all extraction processes in samples that had been thermally processed (100 °C and 120 °C). This data showed that the varying degree of processing affects the yield and integrity of DNA in highly processed products. A "weight ladder" (a mix of fragments of known size) which serves for estimation of the size of the isolated DNA was seen on certain strips. No fragment above 10 kb was observed. According to Zvarová et al. (2012), this is evidence of the importance of the selection of correct isolation procedures and differences in quality between individual manufacturers of isolation kits. Nevertheless, a better option is to compare the integrity of the isolated DNA by direct checks on PCR reactions. The results of PCR amplification from extracts of meat products showed that significant differences exist in the performance of each method. Specific results from all 25 model samples are depicted in Table 3 and Figures 2 – 9. It is hard to compare our results with other studies in view of the inadequate specialist literature of a similar nature.

Table 3. PCR amplification (primers according to Matsunaga et al. 1999)

Samples	Kit A			Kit B			Kit C			Kit D			Kit E			Kit F			Kit G			Kit H		
	S	G	B	S	G	B	S	G	B	S	G	B	S	G	B	S	G	B	S	G	B	S	G	B
1	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0
2	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0
3	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0
4	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	-	0	0	+	0	0
5	+	+	0	+	+	0	-	+	0	+	+	0	+	+	0	+	+	0	-	+	0	+	+	0
6	+	+	0	+	+	0	-	+	0	+	+	0	+	+	0	+	+	0	-	+	0	+	+	0
7	+	+	0	+	+	0	-	+	0	+	+	0	+	+	0	+	+	0	-	+	0	+	+	0
8	-	+	0	+	+	0	-	+	0	-	+	0	-	+	0	-	+	0	-	+	0	-	+	0
9	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0
10	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0
11	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0
12	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0
13	+	+	0	+	+	0	+	+	0	+	+	0	+	+	0	+	+	0	-	+	0	+	+	0
14	+	+	0	+	+	0	+	+	0	+	+	0	+	+	0	+	+	0	+	+	0	+	+	0
15	+	+	0	+	+	0	+	+	0	+	+	0	+	+	0	+	+	0	-	+	0	+	+	0
16	+	+	0	+	+	0	-	+	0	+	+	0	-	+	0	-	+	0	-	+	0	-	+	0
17	+	+	-	+	+	-	-	+	+	+	-	+	+	+	-	+	+	-	+	+	-	+	+	-
18	+	+	-	+	+	-	-	+	+	+	-	+	+	+	-	+	+	-	-	+	-	+	+	-
19	+	+	-	+	+	-	-	+	+	+	-	+	+	+	-	+	+	-	-	+	-	+	+	-
20	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0
21	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0	+	0	0
22	+	0	+	+	0	+	-	0	-	+	0	-	+	0	-	+	0	+	+	0	+	+	0	+
23	?	+	+	-	+	+	-	-	+	+	-	+	+	0	-	+	+	-	-	+	+	+	+	-
24	-	+	0	-	+	0	+	-	0	+	-	0	-	+	0	-	+	0	-	+	0	-	+	0
25	+	+	-	+	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	+	-	-	+	-

S – *Sus scrofa domesticus*, G – *Gallus gallus*, B – *Bos taurus*

+ positive, - negative, 0: should not be detected

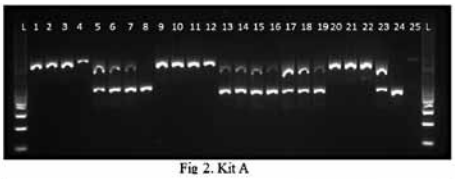
Fig. 2. Kit A

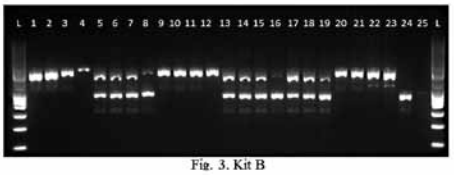
Fig. 3. Kit B


Fig. 4. Kit C


Fig. 5. Kit D


Fig. 6. Kit E

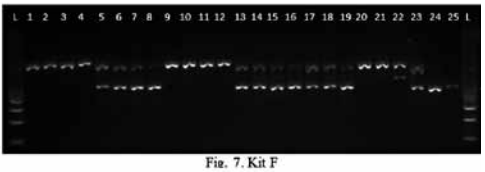
Fig. 7. Kit F


Fig. 8. Kit G

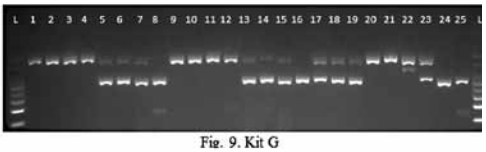
Fig. 9. Kit G

In the final phase of the study, the overall financial costs and manual demands of work during extraction using the various kits were assessed (Table 4). Kit B or kit A seem optimal according to the assessment presented in the table.

Table 4. Costs and labour intensity associated with extraction kits

Extraction process	Price per sample[a]	Weight of sample [mg of tissue]	Labour intensity concentration [ng·µl^{-1}]	Average values of DNA 1.7 – 2.0	Range of absorbance [A260 /A280]
A	++	25	+	24	48 / 50
B	+++	200	+	74	46 / 50
C	++	10	++	70	11 / 50
D	+++	200	+	15	24 / 50
E	++	200	+	56	5 / 50
F	++	25	+	33	1 / 50
G	+	100	+++	425	45 / 50
H	+++	200	+	67	22 / 50

[a] 0 – 50 +, 50 – 100 ++, 100 – 150 +++

Conclusions

This study assessed the effectiveness of eight extraction kits and compared the DNA isolation of meat products with different compositions and technological processes. According to the data obtained on the assessment of DNA concentration and purity, kit B (DNeasy mericon Food Kit, Qiagen) would seem the best for DNA extraction.

A comparison of the effectiveness of various kits was also performed with a view to financial cost, the demanding nature of work with them, time demands and the input amount of raw materials – in this regard kit A (DNeasy Blood & Tissue Kit, Qiagen) and kit B are optimal. Kit A and kit B are assessed as suitable on the basis of the most important determination, i.e. PCR analysis.

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