



THE USE OF PCR AND REAL-TIME PCR FOR QUALITATIVE AND QUANTITATIVE DETERMINATION OF POULTRY AND CHICKEN MEALS*

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Abstract

European Union regulations (e.g. Commission Regulation (EU) No 56/2013) set restrictions on the use of poultry meals in animal nutrition, which requires the species composition of manufactured feeds to be constantly monitored. The aim of this study is to develop a method for qualitative analysis of poultry meals, enabling the four main poultry species (chicken, turkey, duck, goose) to be detected using one pair of primers, and a method for quantitative determination of chicken meals in poultry meals. The qualitative identification method was developed using PCR technology, whereas qPCR and TaqMan probes were used for quantitative identification. The study was performed with samples of feed mixture containing poultry and chicken meal. The limit of determination was 0.08% and 0.02% for qualitative and quantitative identification, respectively. The results of quantitative determinations obtained for independent DNA isolations from the same samples are repeatable ($RSDcT \leq 0.46\%$). The determined concentrations are accurate ($Dc \leq 11.23\%$ for $c \geq 0.06$). The identification of target sequences in both tests is good enough for commercial applications.

Key words: poultry meal, species identification, PCR, real-time PCR, mitochondrial DNA

Human and animal nutrition is regulated by a number of laws, which are aimed to protect human health and animal welfare. They give particular attention to the conformity of foods or feeds with description provided by the producer on the label in terms of species composition (EU No 1169/2011, EC No 178/2002) and with the international food recommendations (e.g. EU No 142/2011, EU No 56/2013). The last-mentioned law, which lays down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies, provides information about restrictions on the use of animal meals. Accordingly, it is possible to lift the ban on the use of processed animal protein in non-ruminant feed without lifting the existing prohibition on intra-species recycling provided that validated analytical techniques to determine the species origin of processed animal protein are available.

*Work financed from statutory activity, project no. 07-4.14.7.

These research tools would enable the lawmakers to allow the use of processed animal protein for producing monogastric (pig and poultry) feeds, based on a proposal put forward by the European Parliament to overcome the protein deficit in the EU. In addition, the traceability of poultry, in particular chickens, is very useful when verifying the composition of pet foods because many pets are allergic to chicken. It should also be mentioned that the chicken component is the most popular poultry ingredient due to its low price and widespread availability on the market.

All these arguments speak in favour of the need for species authentication of the commercial feeds available on the market. Unfortunately, there is no approved diagnostic method to detect the presence of pig or poultry material in feed.

Studies on species identification of food ingredients have been carried out for several dozen years, but the most significant developments have occurred over the last two decades (Cheng et al., 2003; Hou et al., 2015; Bottero and Dalmaso, 2011). The most effective methods are based on analysis of mitochondrial DNA (mtDNA).

The present study analysed fragments of 12S rRNA and 16S rRNA. Both have been extensively described in the literature as useful for routine determination of the species origin of meat. 12S rRNA and 16S rRNA loci sequences are highly conservative in different animal species, which enables universal primers to be designed (Karlsson and Holmlund, 2007; Kocher et al., 1989). At the same time, differences in their sequences are sufficient to use them for identification of individual species (Pegels et al., 2015; Mahajan et al., 2011). Both fragments are used in classical PCR, qPCR, or sequencing. As regards the identification of poultry components, numerous methods for qualitative (Mahajan et al., 2011; Farag et al., 2015; Abuzinadah et al., 2013) and quantitative (Safdar et al., 2015; Kesmen et al., 2009) determination of individual species are known, for which the limit of determination in meat and meat preparations exceeds 0.1% (Pegels et al., 2012; Soares et al., 2010; Dooley et al., 2004; Karabasanavar et al., 2013). However, this paper presents a method to detect smaller amounts of the components of chicken in meals than those described in the literature methods. It should also be noted that too little attention has been given so far to the comprehensive identification of poultry components, which enables several species of domesticated breeding birds to be detected using one pair of primers.

Therefore, the objective of this study was to develop the methods for:

- quantification of chicken meals,
- qualitative analysis of poultry to detect four poultry species using one pair of primers.

The developed methods were used to test three feed mixtures for the presence of poultry meal.

Material and methods

Tests were performed with samples of:

- porcine meat (*Sus scrofa*), bovine meat (*Bos taurus*), ovine meat (*Ovis aries*), equine meat (*Equus caballus*), chicken meat (*Gallus gallus*), duck meat (*Anas platy-*

rhynchos), goose meat (*Anser domesticus*), and turkey meat (*Meleagris gallopavo*). The meat samples were stored at -20°C until analysis,

- rice, maize, soybean,
- guaranteed analysis feed mixtures without animal meals (KN), and mixtures containing poultry, ovine, bovine and porcine meals in the amount of 5%. In addition, 0.2% poultry meal obtained by combining appropriate proportions of 5% poultry meal with KN,
- feed mixture with the total poultry meal content of 100%, 10%, 1%, 0.1%, 0.08%, 0.07%,
- feed mixture with the total chicken meal content of 10%, 1%, 0.1%, 0.06%, 0.04%, 0.02%, 0.01%,
- commercial feed mixtures P1, P2 and P3.

Samples of grain and feed mixtures were stored at $+4^{\circ}\text{C}$ until analysis.

The guaranteed analysis feed mixtures originated from the Proficiency Test (Protein in animal feed/APHA). The meat was obtained from a slaughterhouse (beef, pork, mutton, horse meat) and from a shop (poultry). The meat of different poultry species was purchased in sufficient amounts to enable morphological assessment of species identification. Soybean, rice and feed mixtures P1, P2 and P3 were also sourced from trade. Soybean and rice were organically certified. Only the plant components (including barley, sugar beet pulp, sunflower meal, soybean meal, rapeseed meal, oats) of the samples were declared. The poultry and chicken meals were obtained from a manufacturer of pet food ingredients and a feed manufacturer. They were produced in accordance with EU law (133°C , 3 bar, 20 minutes) and their quality was demonstrated by the HACCP system implemented by the manufacturer. Also, before experiment these samples were tested relative to sample of guaranteed composition using a commercial method used in lab (Natonek-Wisniewska et al., 2013).

DNA isolation

Total DNA was obtained with the AX Food kit (A&A Biotechnology), using the isolation method specified by the manufacturer, and finally suspending DNA in 50 μl of TE buffer (Tris-EDTA). DNA concentration was measured with a Nanodrop spectrophotometer while determining the purity of extracted DNA by measuring the 260/280 absorbance ratio.

Designing of primers

The method for identification of the four poultry species (chickens, geese, ducks, turkeys) was elaborated with PCR (Polymerase Chain Reaction) technology, whereas qPCR (Quantitative Polymerase Chain Reaction) was used for quantitative identification. PCR primers specific to poultry were designed using Primer-BLAST software, while the primers and TaqMan probe used for quantitative analysis were designed using Primer Express software (Life Technologies). The probe was labelled with FAM reporter dye. The sequences of the designed primers and probe, the amplicon size are listed in Table 1. The primers were examined using BLAST (Altschul et al., 1997; Ye et al., 2012) to confirm their specificity and to select species whose

DNA is homologous to the proposed primers, thus theoretically giving false positive reactions. Primer-BLAST (Altschul et al., 1997; Ye et al., 2012) was used to check for possible amplification of DNA from the species of plants and animals that are most often used in feeds and food products (cattle, pig, sheep, horse, rabbit, trout, tuna, soybean, rice, maize), animals foreign to European culture (dog, cat), which are a frequent component of banned (mainly folk medicine) products, as well as human DNA.

Table 1. Sequences of the designed primers and probe as well as the annealing temperature

Species identified	Primers /probe	Sequence (5'–3')	Annealing temperature
Poultry (chicken, goose, duck, turkey)	forward	GAGAACTACGAGCACAAACGCTT	56°C
	reverse	AGAACAGGCTCCTCTAGGTGGGT	
Chicken	forward	Natonek-Wiśniewska et al., 2013	63°C
	reverse	Natonek-Wiśniewska et al., 2013	
	probe	CCCTCCCCGCACTG	

In addition, bioinformatic analysis of target DNA sequences for poultry showed that the selected segments (1800→1874 for Nc_001323; 594→668 for Nc_011196; 570→644 for Nc_010195; 1638→1712 for Nc_009684) were 100% (or 99%) homologous to each other. What is more, none of the selected sequences used in both analyses are found in the genome of any plant and animal species potentially occurring near those determined in feeds and food products.

Development and optimization of PCR assay and determination of method parameters

A kit containing HotStarTaq DNA Polymerase (Qiagen Cat. No. 203 203), 0.6 pM of each primer, 200 µM of each dNTP, nuclease free water and 400 ng DNA were used for analysis. The concentration of the other reagents followed the manufacturer's instructions. Total volume of the reaction mixture was 22.5 µl. Thermal program dedicated by HotStarTaq DNA Polymerase manufacturer was used. Annealing temperature is shown in Table 1. Species specificity of the primers was tested using DNA from the samples of meat, rice, maize, soybean, a feed mixture containing 5% poultry, bovine, porcine and ovine meals, 0.2% poultry meal, and a feed mixture without animal meals (KN). The limits of detection were set based on analysis of the feed mixture containing poultry meal at 100%, 10%, 1%, 0.1%, 0.8% and 0.7%.

Development and optimization of real-time PCR and determination of method parameters

The reaction was performed in 50 µl of TaqMan® Universal PCR Master Mix (Life Technologies, cat. no. 4304437) using the standard thermal programme. The analysis was performed at Step One Plus Real-timePCR. The optimum amounts of

300 nmol primers, 150 nmol probe and 150 ng DNA were used. Linearity of the reaction was calculated from the standard curve, determined from the samples of feed containing 10%, 1.0%, 0.1% and 0.01% of chicken meal, respectively. Each sample was analysed in triplicate for independent isolations. To determine accuracy of the method and limit of determination, the samples containing 10%, 1%, 0.1%, 0.06, 0.04%, 0.02% and 0.01% of the chicken meal were analysed five times to determine their concentration in relation to the standard curve.

Analysis of the commercial feed samples

Both developed methods were used to analyse the commercial samples of feeds P1 and P2. In addition to the negative control, the positive control (PTC), i.e. a reaction with DNA being a mixture of isolates from chicken, duck, goose and turkey meat, was used for PCR of the samples P1 and P2.

Results

The results of the experiment have confirmed that the AxFood kit is suitable for isolation of DNA from feed, because its quantity and quality made it possible to use them in further study. Moreover, DNA samples extracted from processed tissues migrate in the form of a strong band during electrophoresis (Natonek-Wisniewska et al., 2013).

The DNA isolates had a DNA concentration of about 500 ng/ μ l when obtained from the meat and within 250 ng/ μ l when obtained from feed; in both cases, the A260/280 parameter ranged between 1.7 and 2.0. The coefficient of repeatability for independent DNA isolations of the same sample ranged from 0.9 to 1.1, while the difference in their purity did not exceed 4%.

In silico analysis of the designed primer pairs showed their specificity. They bind to the sequences of target species for which they were designed, but do not react with the DNA from 13 other species of plants and animals (mentioned in the Material and methods section), which are potential components of feed mixtures, food products and pseudo-medical products. Although both pairs of primers may attach to the DNA of the wild fowl found in China, Tibet, India and America, this should have no effect on the result of the analysis because there is negligible risk of the components from these species being found in feeds and foods in our cultural area.

The experimentally confirmed specificity of the poultry primers is shown in Figure 1 (A and B). It shows the result of amplification of DNA isolated from the samples of bovine, porcine, lamb, equine, chicken, goose, duck and turkey meat as well as samples of rice, maize and soybean and commercial feed mixtures. The negative control (NTC), namely water amplification, and the positive control from DNA isolated from chicken meat (PTC) were performed for the reaction. The amplification products were only obtained for poultry – chicken (9), duck (10), goose (11) and turkey (12) samples (Figure 1A) and poultry mixtures (13, 14, 24) (Figure 1B).

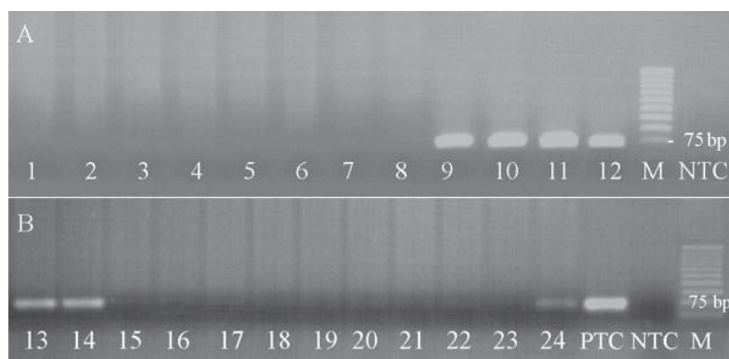


Figure 1 (A and B). Electrophoretic separation of PCR products in 3% agarose gel for species specific reactions and for cross-reactions. The lanes contain PCR product for DNA isolated from: porcine meat (1), bovine meat (2), ovine meat (3), equine meat (4), rice (5), maize (6), soybean (7), feed mixture without animal meal (8), chicken meat (9), duck meat (10), goose meat (11), turkey meat (12), feed mixture without animal meals – KN (21,22,23), feed mixture containing 5% poultry (13,14), bovine (15,16), porcine (17,18), and ovine meals (19,20); and 0.2% poultry meal (24). NTC – negative control of PCR, PTC – positive control of PCR. M – 25 bp size marker (Promega)

The limit of determination for the poultry meal means that the smallest amount that can be determined with this method is 0.08% of the analysed sample (Figure 2). The photograph showed the relationship between band intensity and poultry meal content of the analysed sample (from 0.07% to 100%). The intensity of the result is similar for a given content of the meal and decreases with the decreasing content.

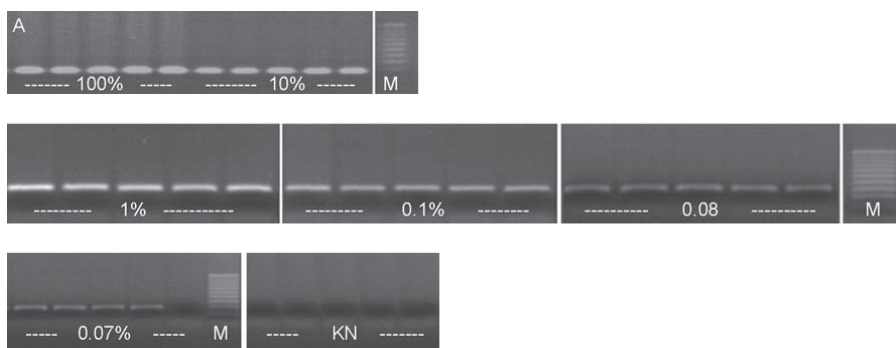


Figure 2. Electrophoretic separation of PCR products across the method range. Lanes contain PCR product for DNA isolated from poultry meal at 100%, 10%, 1%, 0.1%, 0.08%, 0.07% and KN (feed sample without animal components), M – 25 bp size marker (Promega)

The results of analysis of samples P1, P2 and P3 show that the poultry component was only present in samples P2 and P3.

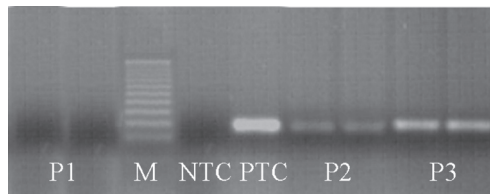


Figure 3. Qualitative analysis of the commercial feed samples. P1, P2, P3 – commercial feed samples, NTC – negative control of PCR, PTC – positive control of PCR. M – 25 bp size marker (Promega)

As expected, the length of the product obtained for all the poultry species analysed and for samples P2 and P3 was 75 bp.

The range of the method ranges from the limit of determination to 100% poultry meal.

Likewise, analysis of the primers used for the quantitative determination (which had been tested in an earlier experiment in cross-reaction with DNA from species such as cattle, sheep, horse, pig, goat, dog, cat, trout, human, rice, maize) demonstrates their species specificity. A positive result was only ascertained for chicken DNA (Natonek-Wiśniewska et al., 2013).

Real-time PCR showed that the amplification occurred for the entire range of the concentration of the determined component (from 0.1% to 10%) (Figure 4). The relationship between cT (threshold cycle) and the concentration of the determined DNA is linear, with the regression coefficient $R^2 = 1$ (Figure 4). cT values for independent isolations are similar, and their relative standard deviation (RSD) is not greater than 0.46% (Table 2).

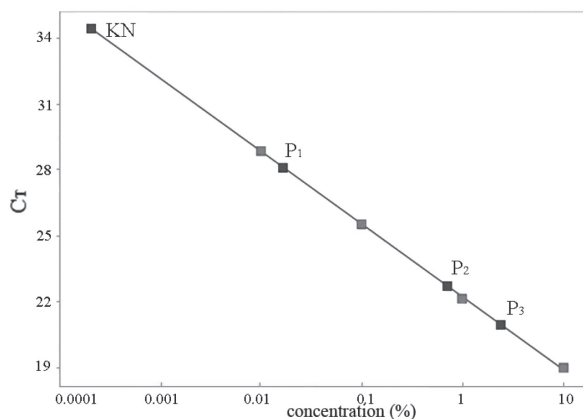


Figure 4. Standard curve for the quantitative determination of the chicken component. P1, P2, P3 – commercial feed samples. KN – feed sample without animal components

The limit of determination (LOD), set at 95% confidence level, was 0.02%. A positive amplification result was obtained for all the samples containing ≥ 0.02 chicken meal. In one case no reaction product was obtained for a concentration of 0.01%.

The presented method was used to determine the concentration of samples with the same composition as standard samples. The values obtained are shown in Table 2. Analysis of the commercial samples shows a concentration of 0.02% for P1 sample, which is equal to the limit of determination. In turn, P2 and P3 samples contained the meal identified at the level of 0.73% and 2.35%, respectively. The analysis performed with both methods confirmed the positive result for P2 and P3 samples, while the qualitative analysis yielded a negative result for the first sample because the addition of 0.02% meal is below the limit of determination in the qualitative method.

Table 2. Results for analysis of standard samples and tested samples (the same as standard as well as commercial ones)

Standard samples (%) *	Tested samples (%)*	Mean c_T	RSD c_T (%)	c (%)	Dc (%)
10		19.03	0.41		
1		22.09	0.29		
0.1		25.58	0.37		
0.01		28.77	0.46		
	10			9.94	0.60
	1			1.15	1.53
	0.1			0.11	6.93
	0.06			0.051	11.23
	0.04			0.03	27.93
	0.02			0.011	32.55
	P1			0.02	
	P2			0.73	
	P3			2.35	

* proportion of chicken component in the feed.

c (%) – identified proportion of chicken component in the feed.

RSD c_T (%) – relative standard deviation c_T for three independent isolations of DNA.

Dc (%) – absolute error of the determined concentration.

Discussion

The aim of the study was to design new tests for qualitative determination of poultry DNA and for quantitative determination of chicken DNA, which could be successfully used to analyse feed mixtures. The qualitative determination was performed by PCR using 12S rRNA fragment homologous to chickens, geese, ducks and turkeys. The quantitative determination of chicken was conducted using 16S rRNA fragment specific for chickens, amplified in qPCR reaction (Natonek-Wiśniewska et al., 2013).

The range of both methods is very broad. The qualitative method ranges from the limit of determination (LOD) to 100% poultry meal. For the quantitative method,

only the fragment between the limit of determination and 10% poultry meal was experimentally tested. A higher range of concentrations was tested by extrapolating the standard curve to the 100% concentration value. So determined cT assumed real values (cT = 17.01).

The LOD obtained during the qualitative determination indicates that the developed method is characterized by the highest sensitivity of all techniques described to date; earlier publications reported that poultry meal can be identified by classical PCR only from the level of 0.1% (Martín et al., 2007).

Both methods are characterized by low cross-reactivity. No falsely positive reactions were noted for the poultry identification method, while for chickens it was observed beyond the 34th cycle in the reaction of DNA from the feed mixture containing no animal meals (KN). However, this has no effect on quality of the analysis, because the falsely positive amount of chicken meal determined for this sample is 0.0002, which is much below the LOD.

mtDNA identification of poultry has been well researched. The methods described in the literature demonstrate that individual species of domesticated fowl can be detected separately (Martín et al., 2007) or in multiplex (Dalmasso et al., 2004; Bottero and Dalmasso, 2011; Okuma and Hellberg, 2015). For example, two species can be identified at the same time, such as duck and chicken (Haunshi et al., 2009) or chicken and turkey (Dooley et al., 2004; Dalmasso et al., 2004). However, the method presented here is more comprehensive because it analyses the four major representatives of poultry (chickens, ducks, geese, turkeys) using one pair of primers, which significantly reduces the costs of analysis.

The second developed test allows for quantitative analysis. A standard curve prepared from low concentration samples was used in the test to obtain a better fit to the analysed samples. Recent literature provides information that quantitative evaluation of the analysed ingredient is not possible when mtDNA is used (Ballin et al., 2009). The present work showed that when the standard curve is prepared from samples with high affinity to the identified samples in terms of type, processing and amount of component, percentage content can be determined with high accuracy, with relative error of less than 11.23% for concentrations of $\geq 0.06\%$, and $\leq 32.55\%$ for lower concentrations (Table 2). A similar regularity was observed by Pegels et al. (2012), who reported that quantitative capability of the assay is limited by the variability in terms of composition and processing treatment of the feeds, which affect the amount and quality of amplifiable DNA.

In both methods, primers generating short amplicons were used (75 bp for poultry and 66 bp for chickens), whereas most of other studies describe the techniques applying to long DNA fragments (Haunshi et al., 2009; Ghovvati et al., 2009). However, the use of short fragments enabled degraded material to be identified, and such DNA chains dominate in highly processed (high pressure and temperature) poultry meals (Frezza et al., 2003).

In summary, the present results show that the developed tests are suitable for analysing feed mixtures for the content of poultry and chicken meals. The presented methods provide a valuable tool for rapid qualitative and quantitative identification of poultry and chicken components.

Both tests proved sensitive and identify target sequences well enough for commercial applications. They also conform with EU directives (EU No 51/2013) and EURL-AP (European Union Reference Laboratory for Animal Proteins) requirements which state that the methods for species identification of animal protein in feed must ensure the minimum identification level of 0.1%.

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Received: 22 VI 2015

Accepted: 29 I 2016