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Analytical Methods

Species identification and quantification in meat and meat products using droplet digital PCR (ddPCR)

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ABSTRACT

Species fraud and product mislabelling in processed food, albeit not being a direct health issue, often results in consumer distrust. Therefore methods for quantification of undeclared species are needed. Targeting mitochondrial DNA, e.g. *CYTB* gene, for species quantification is unsuitable, due to a fivefold inter-tissue variation in mtDNA content per cell resulting in either an under- (−70%) or overestimation (+160%) of species DNA contents. Here, we describe a reliable two-step droplet digital PCR (ddPCR) assay targeting the nuclear *F2* gene for precise quantification of cattle, horse, and pig in processed meat products. The ddPCR assay is advantageous over qPCR showing a limit of quantification (LOQ) and detection (LOD) in different meat products of 0.01% and 0.001%, respectively. The specificity was verified in 14 different species. Hence, determining *F2* in food by ddPCR can be recommended for quality assurance and control in production systems.

1. Introduction

The declaration of meat products in the EU is mandated by the Commission Directive 2002/86/EC stating that meat products have to be labelled with precise information about the species and its percentage in the product (Commission Directive, 2002). Falsely declared meat products have shaken the consumers' acceptance of meat products in the past and represent a wide spread problem which is known for several decades. It was postulated that the overall rate of substituted species was 16.6% in raw and cooked meat samples (Hsieh, Woodward, & Ho, 1995). Furthermore, it was shown that 22.0% of the analysed meat products were not in compliance with the specific legislation (Ayaz, Ayaz, & Erol, 2006). Methods to control meat products have aimed to detect either species-specific proteins (ELISA, liquid chromatography (LC), high performance LC, ultra-performance LC) or DNA (hybridization, PCR, single strand conformational analysis, conformation sensitive gel electrophoresis, RFLP) (Ballin, Vogensen, & Karlsson, 2009; Giaretta, Di Giuseppe, Lippert, Parente, & Di Maro, 2013). DNA detection methods target either single copy genes (Laube, Zagon, & Broll, 2007; Laube et al., 2003) or mitochondrial DNA (mtDNA) (Karabasanavar, Singh, Kumar, & Shebannavar, 2014; Matsunaga et al., 1999). Methods based on mtDNA are highly sensitive. However, since the amount of mtDNA shows higher tissue-specific variation than nuclear DNA, quantification cannot be based on mtDNA instead a single copy chromosomal target is desirable (Ballin et al., 2009). In general, quantification results should be based on genome/genome equivalents and not on weight/weight, because of differences in tissue composition, species genome size, DNA degradation, and extractability (Ballin et al., 2009). Due to the horsemeat scandal, which has evolved into a pan-European, if not a global food fraud in 2013, the detection of minute species admixtures in processed food products has recently become an important diagnostic challenge. Different qPCR assays were developed to distinguish between various amounts of species in processed and several canned foods including beef, pork, lamb, goat, chicken, duck, and turkey (Laube et al., 2007). The reported qPCRs amplify regions in different single copy genes and use external standards for quantification with an absolute limit of quantification (LOQ) in the range of 10–100 genome copies. A method for utilisation under tropical conditions (Laube et al., 2007) facilitated quantification $\geqslant$1% (w/w) and in low-processed meat products as well as in normal canned foods $\geqslant$0.1% (w/w).

An approach to detect traces of DNA is the use of digital PCR (dPCR). It permits counting of single template molecules by separation of extremely diluted nucleic acids into individual reaction compartments, which are monitored for a positive amplification after end-point PCR amplification using fluorescent target-specific hydrolysis probes. The benefits of dPCR in comparison to quantitative

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PCR are its higher sensitivity and precision as well as providing an absolute measure of nucleic acid concentration (Hindson et al., 2011) without the use of standard curves (Pinheiro et al., 2012). External standards for absolute quantification especially in highly processed meat products are problematic, because DNA amplification efficiencies and qualities might vary between the reference and the test sample (Ballin et al., 2009). The precision of qPCR is limited because it cannot reliably distinguish differences that are smaller than 2-fold and quantification of minute minor concentrations (<1%) is often inaccurate. In contrast digital PCR can detect ⩽30% difference in gene expression, distinguish whether a variant occurs in five versus six copies and identify rare variant contents of ⩽0.1% (Baker, 2012). DPCR is more tolerant to inhibitors than qPCR (Hoshino & Inagaki, 2012) and because of partitioning in separate reaction chambers it is robust against many factors that can influence PCR like cross-reacting DNA templates and primer-dimers (Nakano et al., 2003). In droplet digital PCR (ddPCR) each reaction is randomly distributed in several thousands nanoliter-scale water in oil droplets. The absolute number of target molecules initially present in the original sample can be determined by the ratio of positive to total number of droplets (Morisset, Stebih, Milavec, Gruden, & Zel, 2013). Using droplet digital PCR, templates of low concentration can be detected in a background of high numbers of non-target nucleic acids (Morisset et al., 2013; Pohl & Shih Ie, 2004), thus a direct relative quantification is possible.

The aim of this study was the establishment of a new method for exact quantification of different species in meat and processed meat products using droplet digital PCR for routine use in laboratories.

2. Materials and methods

2.1. Test material

Beef, pork, and horsemeat from local meat sellers were cooked and subsequently minced. Different meat mixtures of (0.01–50%) of the species combinations horse:cattle and horse:pig were prepared in a total mass of 100 g. DNA extracted from beef, pork, and horsemeat was mixed in the ratios of 0.1%, 0.01% and 0.001% of the species combinations horse:cattle, horse:pig, cattle:pig and pig:cattle.

Bovine tendon, fat, liver, and muscle tissue were used to determine differences between the mtDNA content.

Processed meat products (Lasagne, Pelmeni, Beef goulash, Swabian pockets) were washed and pieces of meat separated. DNA was isolated from meat products, cold meat (Thuringian ground pork, spiced ground pork, fine salami, coarse salami, onion sausage) and from seven calf liver sausages.

DNA isolated from blood or meat of 14 different species (Suppl. Table S1) and 17 bovine, 26 equine and 6 porcine breeds, including 1 crossbreed (Suppl. Table S2) was used to verify the species specificity of the assays and to rule out breed effects.

2.2. DNA extraction

DNA was extracted from test material using a modified cetyltrimethylammonium bromide (CTAB) method (Amtliche Sammlung von Untersuchungsverfahren nach § 64, 2007). Ten individual portions (200 mg) of each sample were mixed thoroughly with 1 mL extraction buffer solution (20 g/L CTAB, 1.4 mol/L NaCl, 0.1 mol/L Tris·HCl (pH = 8.0), 20 mmol/L Na_2EDTA), 40 μL proteinase K solution (20 mg/ml) and 20 μL RNase A solution (10 mg/mL). The samples were incubated at 65 °C overnight and centrifuged for 10 min at 14,500×*g*. 1 mL of the supernatant was transferred into 570 μL chloroform-isoamyl alcohol (24:1), mixed and centrifuged at 14,500×*g* for 15 min. The upper phase was mixed with 800 μL isopropanol, incubated for 1 h at −20 °C and centrifuged for 15 min at 14,500×*g*. The supernatant was discarded and the pellet was washed with 500 μL ethanol (70%). After centrifugation at 14,500×*g* for 5 min the pellet was resolved in 25 μL nuclease- and protease free water (Braun, Melsungen, Germany) and DNA aliquots were pooled. DNA concentrations were determined fluorometrically using Quant-it™ ds DNA Assay Kit (Life Technologies, Eugene, Oregon, USA) and a GENios Pro microplate reader (Tecan Deutschland GmbH, Crailsheim, Germany) and subsequently verified using ddPCR.

2.3. Droplet digital PCR and analysis

For ddPCR, specific primer pairs and hybridization probes for the mitochondrial cytochrome B gene (*CYTB*) and the chromosomal coagulation factor II gene (*F2*) were designed and purchased from Eurofins MWG Operon (Ebersberg, Germany). Primer pairs for the *CYTB* gene amplified fragments of 151 bp (horse), 146 bp (cattle), and 147 bp (pig) and for the *F2* gene fragments of 95 bp (horse), 96 bp (cattle), and 97 bp (pig) (Suppl. Table S3).

Before performing ddPCR 50 ng, 100 ng, and 1 μg genomic DNA was digested using 10U *Bam*HI (New England Biolabs GmbH, Frankfurt/Main, Germany) in 11 μL 1× ddPCR supermix (Bio-Rad, Munich, Germany) containing 110 ng BSA for 1 h at 37 °C.

After digestion, 900 nmol/L of each primer, 250 nmol/L of hydrolysis probes and 1 nmol/L dUTPs (Carl Roth, Karlsruhe, Germany) were added to the restriction mixture and supplemented with nuclease- and protease free water (Braun, Melsungen, Germany) to a final volume of 22 μL. Droplets were generated using the QX100 droplet generator (Bio-Rad, Munich, Germany) according to manufacturer's instructions. PCR amplifications were performed in a thermal cycler (Biometra, Göttingen, Germany) using the conditions in Table 1, and analysed in a QX100 droplet reader (Bio-Rad, Munich, Germany).

For exact quantification of the minor species fraction in a mixture, 1 μg extracted DNA was used with only the primers and probes of the interrogated species. Primers and probes of both species were used with 50 ng DNA in triplicates for simultaneous detection of the content of two species. Meat mixtures were analysed accordingly, but each sample was analysed in duplicates and triplicates for the diluted samples. On average ddPCR yielded a number of 12,768 accepted droplets with a standard deviation of 1328 droplets.

Poisson statistics were applied to determine the concentration of template molecules in each reaction, confidence intervals are 95% and, if not stated otherwise were calculated according to published formulas (Dube, Qin, & Ramakrishnan, 2008; Whale, Cowen, Foy, & Huggett, 2013).

2.4. Determination of mitochondrial vs nuclear DNA using CYTB and F2 ddPCR assay

To compare *CYTB* and *F2* copies, one DNA sample from either bovine muscle, fat, tendon or liver tissue was extracted. For the

Table 1
Reaction conditions of ddPCR systems.

Step	*CYTB*	*F2*
Initial denaturation (°C/min)	95/10	95/10
Cycles	50	50
Denaturation (°C/s)	95/30	95/30
Primer annealing (°C/s)	55/60	55/10
Primer extension (°C/s)	55/60	68/20
Inactivation (°C/min)	98/10	98/10

CYTB assay DNA was diluted (1:100 and 1:500 (liver)). For the *F2* assay undiluted DNA was used and both ddPCR assays were performed with 7 μL DNA. Additionally, extracted DNA from processed meat products, cold meat, and calf liver sausages were applied in *CYTB* assay with 0.1 ng DNA and *F2* assay with 100 ng DNA. The differences between the DNA quantities are necessary to achieve concentrations within the quantification range.

2.5. Specificity and sensitivity

To test sensitivity of each species-specific assay to different breeds a ddPCR with 50 ng DNA from different horse-, cattle-, and pig breeds (Suppl. Table S2) were performed. Specificity of the assays was tested using DNA from different species and breeds that were tested with all primers and probes for horse, cattle, and pig (Suppl. Table S3).

The limit of quantification (LOQ) was determined by evaluating the repeatability over the dynamic range of ddPCRs conducted using different DNA mixtures (0.001–0.1% minor fraction) and meat mixtures (0.01–50% minor fraction). Repeatability was assessed by calculating the coefficient of variation of the measured percentages from nine ddPCR-measurements conducted on consecutive days. In each run the minor fraction was determined from two repetitions. Three repetitions performed in one run were used for the meat mixtures.

The limit of detection (LOD) was defined as the lowest concentration that could be stably detected in all ddPCRs.

3. Results and discussion

3.1. Determination of mtDNA using CYTB ddPCR assay

Mitochondrial DNA (mtDNA) is a well-established target for detection of traces in mixtures. The *CYTB* gene is highly conserved and the only mitochondrial gene coding for a subunit of respiratory complex III. It has a length of 1140 bp and encodes a single polypeptide with a length of 380 amino acid (OMIM, 2011). *CYTB* is present in all mammals with high copy numbers and is useful for qualitative species detection, which has been shown in various studies (Ballin et al., 2009; Maede, 2006; Matsunaga et al., 1999). The used primers for the *CYTB* assay for cattle, horse, and pig were located in the coding region. As to whether mtDNA can be used for quantification purposes is in doubt, due to the variable content of mitochondria in different mammalian tissues (Ballin et al., 2009; Robin & Wong, 1988), whereas a quantitative determination of this variability, based on precise determinations is missing. To elucidate the differences between mtDNA and nuclear DNA content we conducted ddPCRs of the two targets using one DNA sample from each bovine muscle, fat, tendon and liver tissue as examples. The ratios calculated for CYTB copies versus *F2* copies were 1095 (CI: 1141–1051) for muscle, 184 (CI: 191–178) for fat, 225 (CI: 217–235) for tendon and 935 (CI: 1077–811) for liver. Even testing only one DNA extraction per tissue the already detected 6-fold difference between fat and muscle tissue illustrates the high variability in mitochondrial DNA content. Given that the quantitative determination of the content of a false declared species requires two determinations (one for the declared and one for the undeclared), the error if mtDNA is used can add up to more than 10-fold. The problem of w/w quantification with mtDNA is in particular related to processed food, because it may also consist in part of fat, tendon, and offal in addition to muscle meat. Thus, the quantification of traces of an unwarranted species cannot be achieved by using mtDNA, but rather has to be based on a nuclear gene. For quantification based on nuclear DNA, knowledge of tissue type is not important anymore and it has been proposed to express the result as genome/genome equivalents and not on weight/weight (Ballin et al., 2009) even if genomic DNA is used for quantitative species determinations. This seems to be the most correct way of reporting species traces based on such detection techniques. We used a single copy gene *F2*, since it is present in all mammals and shows enough cross-species variability to ensure species-specific detection.

3.2. Determination of nuclear DNA using ddPCR assay

Species-specific primers were designed for intronic regions of the *F2* gene, which showed more nucleotide differences than the exonic regions within the examined species. The PCR amplicon of the three species cattle, horse, and pig was located in intron 5 and reverse primers are partially in exon 6.

3.3. Specificity, LOQ and LOD

The specificity of the *F2* assay was detected for the 14 species listed in Suppl. Table S1. The sensitivity of the primer/probe combinations was retained in each breed of the tested species listed in Suppl. Table S2. This shows that the assays can be used for reliable quantification of species in mixtures.

The LOQ of the *F2* ddPCR systems was defined as the lowest concentration of mixture with a coefficient of variation (CV) $\leqslant$ 25% for quantification. The CV cut-off was chosen according to the relative repeatability standard deviation suggested in the "FAO Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific proteins in food" (Codex Committee on Methods of Analysis and Sampling, 2010). Based on this criterion the limit of quantification for DNA (Table 2) and meat mixtures is 0.01% (Table 3) with the ddPCR, which is one order of magnitude lower than the LOQ reported with qPCR in low-processed meat products and normal canned food (Laube et al., 2007).

The results of recovery of the respective species spiked in meat mixtures are summarised in Table 3. In the meat mixtures prepared of 50% w/w and 10% w/w horsemeat in beef or pork, 53.56% (SD = 0.61%) and 12.93% (SD = 0.25%) horse DNA were detected, whereas the lower amounts showed a good recovery, in particular with respect of difficulties to homogenise the tissues to ensure a completely representative sampling for DNA extraction. In addition, the accuracy of quantification can be influenced by the degradation of the DNA, resulting from the processing of meat and meat products, the genome size between the species and the degree of ploidy (Laube et al., 2007), e.g. muscle and liver cells could be polyploid by fusion or perturbed mitosis (Davoli & de Lange, 2011). Overall these results should be representative for the performance that can be yielded on a routine-testing basis, where

Table 2
Limit of quantification and limit of detection for DNA mixtures in *F2* ddPCR assays represented as mean values and coefficient of variation in brackets (n = 18).

DNA mixtures (%)	Horse/cattle	Horse/pig	Cattle/pig	Pig/cattle
0.1	0.066% (7.43)	0.106% (4.46)	0.085% (3.85)	0.139% (10.83)
0.01	0.007% (19.90)	0.010% (14.63)	0.006% (11.04)	0.021% (15.68)
0.001	0.0004% (78.95)	0.001% (39.58)	0.002% (21.19)	0.0015% (56.07)

Table 3
Limit of quantification for meat mixtures (tissue/tissue) in *F2* ddPCR assay represented as mean values and standard deviation (%) in brackets.

Meat mixtures (%)	Horse/cattle	Horse/pig
50	53.56% (0.61)	52.24% (0.19)
10	13.14% (0.13)	12.93% (0.25)
1	1.04% (0.03)	0.7% (0.01)
0.1	0.07% (0.0002)	0.1% (0.01)
0.01	0.08% (0.001)	0.01% (0.001)

diverse species proportions can be reliably determined down to 0.001% (LOD) and reliably quantified down to 0.01% (LOQ) for different DNA mixtures (Table 2). Therefore this robust and accurate test system is well suitable for food control measures.

3.4. Test procedure of processed meat products

In the first step a quantitative PCR for each species was performed with standards, prepared from meat mixtures horse:cattle and horse:pig (10%, 1%, 0.1% and 0.01%). If no traces were detectable, the results are based on this first step. Depending on whether the quantity of the suspected trace species was > 1% or less than 1% a one-step or two-step ddPCR assay was performed, respectively. An outline of the experimental procedure is depicted in Fig. 1. For quantification of admixtures > 1% a ddPCR with 100 ng DNA and primers/probes for both present species was carried out. To quantify the minor species fraction of less than 1%, a ddPCR was performed with 1 μg DNA and primer/probes for the species in question. Simultaneously, a ddPCR with 50 ng DNA and primer/probes for both species was implemented to quantify the major species ratio. With at least 5–7 orders of magnitude, the dynamic range in qPCR is wider than in the ddPCR (Gachon, Mingam, & Charrier, 2004; Heid, Stevens, Livak, & Williams, 1996) used here, which covers more than 4 orders of magnitude (Pinheiro et al., 2012). For digital PCR the upper bound of the dynamic range is dependent on the number of individual reaction compartments provided. The lowest imprecision of the applied Poisson statistics is achieved at 80% template-occupancy of the compartments with a sharp rise towards 100% occupancy (Dube et al., 2008). Therefore,

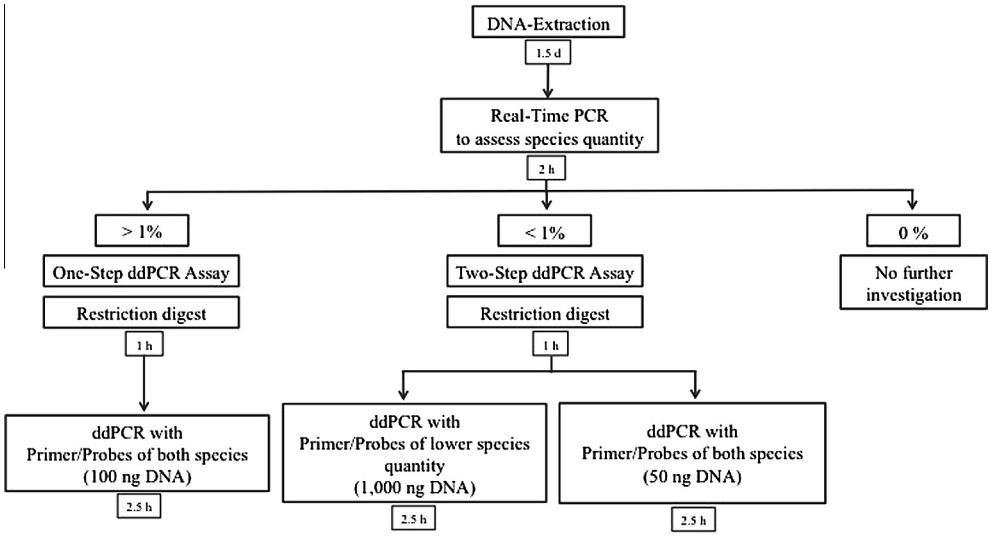

Fig. 1. Workflow for DNA quantification of different species in meat and meat products.

Table 4
Species content in seven calf liver sausages, processed food and cold meat (content in percentage of the meat fraction; 95% confidence interval in brackets).

	Samples	Manufacturer's information (%)		*F2* ddPCR (%)		*CYTB* ddPCR (%)		Δ*CYTB-F2*
		Cattle	Pig	Cattle	Pig	Cattle	Pig	
Calf liver sausages	S1	20.9	79.1	5.9 (5.7–6.1)	94.1 (93.9–94.3)	5.3 (5.1–5.4)	94.8 (94.6–94.9)	−0.2
	S2	20.5	79.5	3.1 (3–3.2)	96.9 (96.8–97)	2.7 (2.5–2.8)	97.3 (97.2–97.5)	−0.3
	S3*	17	83	19.1 (18.7–19.5)	80.9 (80.5–81.3)	22.8 (22.4–23.2)	77.2 (76.8–77.6)	0.2
	S4*	19.5	80.5	6 (5.8–6.1)	94.1 (93.9–94.2)	3.8 (3.7–4)	96.2 (96–96.3)	−0.4
	S5	17.6	82.4	4.7 (4.5–4.9)	95.3 (95.1–95.5)	6.2 (5.9–6.4)	93.8 (93.6–94.1)	0.2
	S6	21.4	78.6	5.3 (5.1–5.5)	94.7 (94.5–94.9)	4.4 (4.2–4.6)	95.6 (95.4–95.8)	−0.3
	S7*	20.3	79.7	6.5 (6.3–6.7)	93.5 (93.3–93.7)	3.7 (3.6–3.9)	96.3 (96.1–96.4)	−0.5
Meat products	Lasagne*	72.6	27.4	86.9 (86.6–87.2)	13.1 (12.8–13.4)	92.4 (92.1–92.8)	7.6 (7.2–7.9)	0.1
	Pelmeni[a]		100		100	–	–	
	Beef goulash[a]	100		100		–	–	
	Swabian pockets[a]		100		100	–	–	
Cold meat	Thuringian ground pork	–	–	0 (0–0.1)	100 (99.9–100)	0 (0–0.1)	100 (99.9–100)	
	Spiced ground pork	–	–	0 (0–0.1)	100 (99.9–100)	0 (0–0.1)	100 (99.9–100)	
	Fine Salami	–	–	0.06 (0.05–0.07)	99.94 (99.93–99.95)	0.12 (0.1–0.15)	99.88 (99.85–99.9)	1.0
	Coarse Salami	–	–	0.12 (0.11–0.15)	99.88 (99.85–99.89)	0.04 (0.03–0.06)	99.96 (99.94–99.97)	−0.7
	Onion sausage*	–	–	1.2 (1.1–1.3)	98.8 (98.7–98.9)	3 (2.9–3.2)	97 (96.8–97.1)	1.6

ΔCYTB-F2 were calculated by dividing the difference between the determined percentages of cattle CYTB and F2 ddPCRs by the *F2* value.
[a] Quantification based on real-time qPCR.
* Significant difference between F2 and CYTB ddPCR ($p < 0.05$).

the upper limit of template molecules for a ddPCR with app. 20,000 droplets is app. 16,000 template molecules to yield the highest reliability. The bounded dynamic range in ddPCR was overcome by a two-step assay for quantification of minor species $\leqslant$ 1%, since otherwise enough positive droplets for the lower species content and enough negative events for the major content cannot be achieved simultaneously in one reaction. Since the dynamic range of target DNA quantification in dPCR, directly depends on number of partitions (Hindson et al., 2011), dPCR methods providing more reactions may be capable to precisely quantify the minor fraction over the entire needed range in one step.

Using this analytical concept, the respective species content in processed meat products and calf liver sausage samples (run in duplicates) was investigated and the data are summarised in Table 4. Compared to the manufacturer's declarations for beef content in calf liver sausages, quantification using the *F2* ddPCR procedure showed a significant lower content in six of the seven products and a significant higher content in one sausage ($p < 0.0001$). The content of cattle DNA varied between 3.1% and 19.1%. The onion sausage sample and the tested Lasagne showed a higher percentage of beef DNA, compared to the manufacturer's content information ($p < 0.0001$). No equine DNA was detectable in any of the tested processed meat products (Table 4). When the beef content determination based on genomic and mitochondrial DNA was compared, the use of mitochondrial DNA tended to over- and underestimate the DNA content with a range of −70% to +160% (ΔCYTB-F2 – Table 4).

4. Conclusion

Due to several food scandals within the last years, the necessity of a cost effective and reliable method for quantification of animal species in meat and processed meat products has become a major challenge. From the data presented here, it can be concluded that the use of mtDNA cannot be recommended due to an at least 5-fold variability between different tissues (fat versus muscle) compared to nuclear DNA. This variability is even more pronounced if weight/weight percent concentrations are used. Therefore the use of nuclear genes and a genome/genome equivalent ratio is advantageous. In addition, the number of different nuclear genes and/or nuclear DNA compared to mtDNA allows the development of an almost infinite number of highly species specific assays. The combination of a qPCR and ddPCR two-step assay allowed the reliable species quantification (LOQ) and determination (LOD) in production-related admixtures and processed foods with 0.01% and 0.001%, respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.10.138.

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