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Analytical Methods

A multiplex real-time PCR method for the quantification of beef and pork fractions in minced meat

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ABSTRACT

One popular staple food in many lands is minced meat, traditionally prepared from beef and/or pork fractions. While beef is the more expensive of the two meat fractions, the possibility exists for manufacturers to fraudulently declare higher proportions of it. Additionally, the need exists to protect consumers who, out of medical or ethical reasons, reject specific meat fractions.

In this work, we report on a quantitative triplex real-time PCR approach for the quantification of meat in minced meat products. With the method, beef and pork fractions are quantified employing primer and probe sequences that specifically recognise cow and pig components, against the backdrop of myostatin, a universal sequence commonly found in mammals and poultry species. The limit of detection of the qPCR method was 20 genome equivalents, while the measurement of uncertainty was determined at 1.83%. The method was validated on several commercially available minced meat products and performed well in terms of handling, reproducibility and robustness.

1. Introduction

An integral part of the duties of a food control agency is the routine surveillance of food products, including meat-based edibles to ensure that their actual composition correlate with declared components. Meat products comprise a significant proportion of the protein intake of millions worldwide, with global consumption of meat rising steadily. While demand for beef was at its peak in the early 60s', accounting for 40% of the global meat consumption, its dominance has declined steadily, with consumption falling to 23% in 2007. Pork accounts for the most commonly consumed meat fractions today, partly because of its relative cheapness, abundance, and lower production costs (The Economist Online, 2012).

Authentic declaration of meat products may be particularly important to several members of the community, for example individuals who as a result of religious persuasions or health reasons, reject certain types of animal fractions (Ali, Hashim, Sabar Dhahi, Mustafa, & Bin Che Man, 2012). Additionally substitution of more expensive meat with cheaper derivatives might violate consumer trust and confidence. The foregoing emphasizes the importance of the implementation of reliable analytical methods by the relevant regulatory bodies for the determination of the exact composition of meat products. Recent scandals like the horse meat scandal that spread across Europe in early 2013, show the importance of analytical tools not only for detection of the meat constellation in a particular product, but also for quantitative determination of the individual components. This is important in distinguishing inadvertent contamination from deliberate adulteration of meat products, with accompanying legal consequences.

PCR-based methods, from singleplex reactions to multiplex systems (mostly real-time PCR assays) have increasingly become relevant in the analysis of food products including meat samples (Girish, Haunshi, Vaithiyanathan, Rajitha, & Ramakrishna, 2013; Mane, Mendiratta, & Tiwari, 2012; Köppel, Eugster, Ruf, & Rentsch, 2012 and Köppel, Daniels, Felderer, & Brünen-Nieweler, 2013). Multiplex PCR reactions offer the distinct advantages of lower costs and expenditures, coupled with a time-saving feature. Such methods however, typically quantify the DNA of the animal species present in the meat product (López-Andreo, Aldeguer, Guillén, Gabaldón, & Puyet, 2012; Eugster, Ruf, Rentsch, & Köppel, 2009; Drummond et al., 2013). While such results are useful, a direct correlation between DNA content and actual meat percentages is more desirable and this may not always be possible considering the complexity of tissues utilised for meat preparations, with accompanying variations in the extractable DNA. For reliable quantification of actual meat contents, reference materials suitable for each meat product under examination would be required. Production of such appropriate meat standards is

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however time-consuming and laborious. Additionally, due to the complexity in the manufacture of several meat products, with accompanying variations in manufacturers' recipes and production style, generating reference materials appropriate for each commercial meat product might not be feasible.

In this work, a multiplex real-time PCR assay for the quantitative determination of beef and pork fractions in minced meat is described. The triplex assay utilizes previously published animal species - specific primers and probes, relative to the proportion of the reference gene myostatin present in most mammals and bird species (Laube, Zagon, Spiegelberg, et al., 2007; Köppel, Ruf, Zimmerli, & Breitenmoser, 2008). The meat contents of the samples are accordingly computed as percentage compositions. Results from comparison of the triplex method with two other previously described assays are presented and discussed.

2. Materials and methods

2.1. Production of reference minced meat samples

For validation of the presented triplex real-time qPCR method, approximately 6 kg of analytically pure beef and pork minced meat were prepared in a professional environment at the Bavarian Health and Food Safety Authority (LGL). 300 g of minced meat fractions derived from varying proportions of beef and pork were produced to cover a dynamic range of 5–95% beef/pork ($50_{beef}/50_{pork}$, $70_{beef}/30_{pork}$, $80_{beef}/20_{pork}$, $45_{beef}/55_{pork}$, $5_{beef}/95_{pork}$) and vice versa in a first series, and a second series of beef and pork mixtures to cover the trace regions of 0.1–2% of beef and pork respectively ($98_{beef}/2_{pork}$, $99_{beef}/1_{pork}$, $99.5_{beef}/0.5_{pork}$, and $99.9_{beef}/0.1_{pork}$ and vice versa). Homogenisation was carried out in a dedicated thermomixer (Thermomix TM21, Vorwerk, Germany) at mode 2 for up to 5 min. Mixtures were typically stored at −20 °C until required.

2.2. Minced meat and other meat products

Additional to the reference minced meat samples described above, the performance and robustness of the presented quantitative triplex real-time PCR was tested on 50 commercially available minced meat samples randomly selected by the official food monitoring and surveillance authority. More than thirty meat products with varying composition and matrices were additionally included to assess transferability of the method to other meat matrices (see Tables 3 and 5).

2.3. DNA extraction

Four grams each of the examined meat samples was subjected to DNA extraction procedures, employing a modified CTAB protocol previously described (ISO 21571:2005, modified). Additionally a commercially available silicon-column based DNA extraction kit (Surefood Animal X Kit, Congen Biotechnology, Germany) was used to extract DNA in parallel from a subset of meat products of other composition. The two extraction methods were compared to determine the suitability and efficiency of the commercial kit against the time-intensive CTAB Extraction protocol. Following DNA extraction, the purity and concentration of the DNA samples were confirmed either by conventional photometry, employing Nanodrop technology (Nanodrop 1000, Peqlab, Germany) or by Picogreen measurement. DNA samples were typically diluted 1:200, resulting in a final template concentration of at least 10 ng pro PCR reaction.

Table 2
The table depicts precision (relative repeatability standard deviation, RSD_r), accuracy and trueness results obtained from analysis of minced meat containing defined proportions of beef and pork. Results were compiled from at least 5 different runs with an average of 21 measurement points or test results.

Actual pork proportion (%)	Measured pork proportion (%)	Precision (RSD_r)%	Accuracy (%)	Trueness (%)
50	51.78	2.79	2.37	2.44
30	33.35	4.88	6.71	7.10
70	65.72	6.12	7.07	4.61
20	21.37	5.61	7.59	4.54
80	75.55	2.48	4.36	4.16
5	5.71	11.56	13.82	11.33
95	93.21	0.28	1.36	1.36

2.4. Primers and probes

The primers and probes described in this work have been previously reported and are listed in Table 1. Beef and pork fractions were quantified over dedicated primer and probe sequences against the backdrop of a universal sequence commonly found in mammals, namely the housekeeping gene myostatin. For each of the three targets the specific TaqMan probe was labelled with a different fluorescence dye (see Table 1). The primer and probe systems applied in this work all target single copy, chromosomally encoded gene sequences. The 6-FAM, HEX and ROX – labelled probes were quenched with a Blackberry quencher (BBQ, TIB Molbiol, Berlin, Germany) on their 3′-end. Preliminary titration experiments were initially carried out to determine the optimal primer and probe concentrations for the multiplex reaction, without negative impact on the sensitivity of the assay.

2.5. Specificity

Specificity of the applied primer and probe constellation is an important prerequisite for any real-time PCR system. Although the primers and probes applied in this work had been previously reported by other workers, an exhaustive specificity test was carried out against the backdrop of several animal and plant species

Table 1
Primer and probe sequences used for the quantitative triplex real-time PCR assay.

Name	Target gene	Sequence 5′–3′	References
Bos-PDE-f	Cyclic-GMP-phosphodiesterase	ACTCCTACCCATCATGCAGAT	Laube, Zagon, Spiegelberg, et al. (2007)
Bos-PDE-r		TGTTTTTAAATATTTCAGCTAAGAAAAA	
Bos-PDE-probe (ROX)		AACATCAGGATTTTTGCTGCATTTGC	
Sus1-F_pork	Beta-actin	CGAGAGGCTGCCGTAAAGG	Köppel et al. (2008)
Sus1-R_pork		TGCAAGGAACACGGCTAAGTG	
Sus1_TMP (HEX)		TCTGACGTGACTCCCCGACCTGG	
My-f	Myostatin	TTGTGCAAATCCTGAGACTCAT	Laube, Zagon, Spiegelberg, et al. (2007)
My-r		ATACCAGTGCCTGGGTTCAT	
My-probe (6-FAM)		CCCATGAAAGACGGTACAAGGTATACTG	