FOOD COMPOSITION AND ADDITIVES

# Quantification of Meat Proportions by Measuring DNA Contents in Raw and Boiled Sausages Using Matrix-Adapted Calibrators and Multiplex Real-Time PCR

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**The quantification of meat proportions in raw and boiled sausage according to the recipe was evaluated using three different calibrators. To measure the DNA contents from beef, pork, sheep (mutton), and horse, a tetraplex real-time PCR method was applied. Nineteen laboratories analyzed four meat products each made of different proportions of beef, pork, sheep, and horse meat. Three kinds of calibrators were used: raw and boiled sausages of known proportions ranging from 1 to 55% of meat, and a dilution series of DNA from muscle tissue. In general, results generated using calibration sausages were more accurate than those resulting from the use of DNA from muscle tissue, and exhibited smaller measurement uncertainties. Although differences between uses of raw and boiled calibration sausages were small, the most precise and accurate results were obtained by calibration with fine-textured boiled reference sausages.**

Meat products like sausages containing ground meat (e.g., hamburger, cevapcici, salami, landjäger = gendarme, mortadella) composed of a combination of pork and beef, sheep, and horse are widely consumed in Europe. With increasing international trade, the determination of meat proportions in these products is an increasing issue for food control laboratories (1). Fraud often involves sausages with incorrectly or undeclared meat combinations, often depending on actual market prices. Pork-free ("halal") products may be contaminated by trace amounts of pork or by higher amounts to improve the profit or taste. In order to prosecute producers for fraud or bad production practices, analytical methods must be able to accurately quantify all expected meat components for a wide range of complex matrixes.

The accurate measurement of meat proportions of samples is a fundamental problem of DNA-based methods like PCR. PCR-based methods are only able to quantify DNA contents (2–15). But meat proportions according to the recipe have to be assessed. Additionally, the accuracy of DNA-based methods may be impaired when analyzing samples with a variety of tissue types like fatty bacon, fatless meat, or connective tissue. As different tissue types exhibit different concentrations of DNA, the proportional weight of meat in sausages may not correspond to the proportions of species-specific DNA proportions, leading to biased results. Even worse, the exact composition of unknown samples will never be known. Therefore, all expectations and speculations about DNA concentrations of different tissues, influence of production technologies, and loss of DNA during ripening and storage, illustrate the problems, but these considerations will not lead to solutions. Only an experimental approach can evaluate the ability to quantify meat proportions of unknown samples. In an earlier study (14), it was shown that meat proportions can be determined with sufficient accuracy and precision by applying real-time PCR in conjunction with matrix adapted standards These standards must be composed and produced similarly to the unknown samples. In this study, the impact of sausage sample composition and types of calibration material for the determination of meat proportions for two additional species (mutton and horse) was assessed.

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## Experimental

### *Reference Sausages as Calibrators*

To elucidate the difference between mature and boiled meat products, sets of boiled and raw matured reference sausages were produced from the same starting material (Tables 1 and 2). The recipes applied were not traditional, but close to a recipe for Sukuk (type boiled). The reference sausages (10 kg each) were produced by Micarna (Bazenheid, Switzerland). These sausages were examined for their content of water, fat, total protein, connective tissue protein, and muscle protein. The meat for the reference sausage type Landjäger (LJ) was taken after a short cutting process, and therefore exhibited a rougher texture (Table 1). For the boiled reference sausages (BW), the same meat was kept longer in the cutter, delivering a fine and more homogenous texture (Table 2).

In addition, four sample sausages were produced. Sample sausages cevapcici and LJ were also produced by Micarna. The sample sausage salami was produced by ABZ (Spiez, Switzerland) according to a traditional recipe, and the sample

**Table 1. Reference sausages and meat proportions of calibration sausages, type Landjäger (LJ)**

| | Reference sausages[a] | | | | |
|---|---|---|---|---|---|
| Net weight, kg | Kal A LJ | Kal B LJ | Kal C LJ | Kal D LJ | Kal E LJ |
| Beef | 1 | 8 | 22 | 31 | 48 |
| Pork | 31 | 48 | 22 | 8 | 1 |
| Horse | 48 | 31 | 22 | 1 | 9 |
| Sheep | 8 | 1 | 22 | 48 | 31 |
| Curing salt | 2.44 | 2.44 | 2.44 | 2.44 | 2.44 |
| Spices and additives | 10 | 10 | 10 | 10 | 10 |
| | Meat proportions of calibration sausages[b] | | | | |
| Fraction, % | Kal A LJ | Kal B LJ | Kal C LJ | Kal D LJ | Kal E LJ |
| Beef | 1 | 9 | 25 | 35 | 55 |
| Pork | 35 | 55 | 25 | 9 | 1 |
| Horse | 55 | 35 | 25 | 1 | 9 |
| Sheep | 9 | 1 | 25 | 55 | 35 |

[a] Recipe for 100 kg of reference sausages, type LJ (raw sausages), used in this study. Values are given in kg for production of the final 100 kg, taking reduction of the weight during the production process into account.

[b] Meat proportions of calibration sausages, type LJ, excluding nonrelevant proportions (curing salt, spices, and additives).

**Table 2. Reference sausages and meat proportions of calibration sausages, type boiled sausage (BW)**

| | Reference sausages type boiled[a] | | | | |
|---|---|---|---|---|---|
| Net weight, kg | Kal A BW | Kal B BW | Kal C BW | Kal D BW | Kal E BW |
| Beef | 0.7 | 8 | 16 | 23 | 37 |
| Pork | 23 | 37 | 16 | 8 | 1 |
| Horse | 37 | 31 | 16 | 1 | 8 |
| Sheep | 6 | 0.7 | 16 | 37 | 23 |
| Water /ice | 22 | 22 | 22 | 22 | 22 |
| Curing salt | 2.44 | 2.44 | 2.44 | 2.44 | 2.44 |
| Spices and additives | 7 | 7 | 7 | 7 | 7 |
| | Meat proportions of calibration sausages[b] | | | | |
| Fraction, % | Kal A BW | Kal B BW | Kal C BW | Kal D BW | Kal E BW |
| Beef | 1 | 9 | 25 | 35 | 55 |
| Pork | 35 | 55 | 25 | 9 | 1 |
| Horse | 55 | 35 | 25 | 1 | 9 |
| Sheep | 9 | 1 | 25 | 55 | 35 |

[a] Recipe for 100 kg of reference sausages, type BW, used in this study. Values are given in kg for production of the final 100 kg, including water and ice, taking reduction of the weight during the production process into account.

[b] Meat proportions of calibration sausages, type BW, excluding nonrelevant proportions (water, spices, and additives).

**Table 3. Sample sausages and meat proportions of sample sausages**

| | Recipe of sample sausages[a] | | | |
|---|---|---|---|---|
| Fraction, % | Cevapcici | Landjäger | Salami | Sukuk |
| Beef | 47.6 | 23.2 | 5 | 42 |
| Pork | 14.3 | | 48 | 3 |
| Horse | 9.5 | 27.8 | 45 | 3 |
| Sheep | 23.8 | 1.85 | 2 | 28 |
| Lard (pork) | | 39.8 | | |
| Water/ice | 2.4 | | | |
| Curing salt | 0.1 | 2.32 | | 20 |
| Spices and additives | 2.4 | 5.05 | | 4 |
| | Meat proportions of samples sausages[b] | | | |
| Beef | 50 | 25 | 5 | 55 |
| Pork | 15 | 43 | 48 | 4 |
| Horse | 10 | 30 | 45 | 4 |
| Sheep | 25 | 2 | 2 | 37 |

[a] Recipe for 100 kg of four types of sample sausages: cevapcici (raw not ripened product); landjäger (raw ripened product); salami (raw ripened product); and sukuk (boiled product). Values are given in percentages of weight, and include water and ice. Proportions of meat only are shown in Table 3.

[b] Meat proportions of reference and sample sausages, excluding nonrelevant proportions (water, spices, and additives).

sausage type Sukuk was produced by a traditional butcher also according to a traditional recipe. Meat proportions of all sample sausages are compiled in Table 3.

### *DNA Dilution Series as Calibrators*

DNA from muscle tissue was isolated according to the individual protocol by each laboratory. DNA contents were measured spectrophotometrically and mixed in order to create a dilution series enabling the quantification of DNA. This step was performed individually by each laboratory. This heterogeneous approach was chosen because only relative proportions were of interest. It was expected that, as long as the DNA from all four species was extracted and treated equally, absolute differences would become unimportant after the calculations of relative proportions.

### *DNA Isolation*

Each laboratory applied its own DNA isolation method. All participants were asked to determine the concentration of the DNA spectrophotometrically after isolation and to use 100 ng DNA in total as template DNA for the PCR (Table 4).

### *Multiplex PCR*

A multiplex real-time PCR system determining DNA content of pork, beef, sheep, and horse was established and applied

**Table 4. Methods used by the participating laboratories[a]**

| Data set | Sample size, mg | DNA isolation method | PCR method | Thermocycler | Replicates | Sum of z-scores of all analytes[b] |
|---|---|---|---|---|---|---|
| 17 | 2000 | Wizard | Allhorse | Rotorgen 6000 | 2 | 31.7 |
| 13 | 500 | CTAB+Wizard | Allhorse | Rotorgen 6600 | 2 | 33.9 |
| 1 | 300 | Wizard | Allhorse | Rotorgen 6000 | 2 | 36.0 |
| 4 | 600 | Wizard | Allhorse | Rotorgen 6000 | 2 | 38.0 |
| 20 | 200 | CTAB | Allhorse | MX3000P | 2 | 42.0 |
| 3 | 400+600 | Wizard+Nucleospin food | Allhorse | Rotorgen 6000 | 2 | 43.0 |
| 5 | 400 | Nucleospin food | Allhorse | Rotorgen 6000 | 2 | 43.8 |
| 16 | 3000 | Wizard | Allhorse | ABI7500 | 2 | 44.3 |
| 8 | 200–300 | CTAB+Wizard | Allhorse | Rotorgen 6000 | 2 | 44.6 |
| 19 | 2000 | CTAB | Allhorse | Rotorgen 6000 | 2 | 44.7 |
| 12 | 300 | Wizard | Allhorse | ABI 7500 | 2 | 46.5 |
| 9 | 300 | Wizard | Allhorse | Rotorgen 6000 | 2 | 46.5 |
| 15 | 1000 | Wizard | Allhorse | Rotorgen 6000 | 2 | 50.6 |
| 14 | 200 | Magn. Beads EZ-1 Qiagen | Allhorse | Rotorgen 6000 | 2 | 52.1 |
| 11 | 50 | Modified CTAB | Allhorse | Rotorgen 6000 | 2 | 55.2 |
| 10 | 40–50 | QIAmp DNA minikit | Allhorse | Rotorgen 6000 | 2 | 57.0 |
| 7 | 200 | CTAB+QIAquick | Allhorse | ABI 7500 | 2 | 59.3 |
| 6 | 5000 | CTAB | Allhorse | ABI 7500 | 2 | 64.6 |
| 18 | 2000 | CTAB+Wizard | Allhorse | Biorad | 2 | 71.8 |
| 2 | 300 | Wizard | AllFleisch | Rotorgen 6000 | 2 | 77.9 |

[a] Compilation of the methods used by the participating laboratories. For the DNA isolation, nine different methods or combinations of methods were used. For amplification, two different PCR systems and four different thermocyclers models were applied. Individual values, which differ from the true value by two times the SD of all measurements per category, have z-score of 2. Therefore, a high z-score indicates low accuracy and vice versa. The sum of all z-scores values of each laboratory for all analytes were summarized for each laboratory and delivered the criteria for the ranking.

[b] The z-score for each laboratory and each analyte was calculated as follows: z-score = (value of the sample minus the true value)/(standard deviation of the measured values of all samples of all data sets).

in 19 laboratories prior to this interlaboratory trial. It consists of four PCR systems using differently labeled TaqMan™ probes (Fam, Joe, Rox, and Cy5) to determine DNA contents of all four species in one tube. The material, concentrations, and performance of these PCR systems were published in detail previously (16). One laboratory also applied a different heptaplex PCR system also described previously (15).

### *Measurements*

Measurements were performed by each laboratory individually and in accordance with routine procedures (including functions like slope correction and automatic optimization of the threshold line). The DNA serial dilution for the calibration by DNA was produced by each laboratory individually, whereas calibration sausages were produced for all laboratories at once as described. Each sample was measured only twice. This avoided emphasis of the individual results of each laboratory, reflecting a more realistic situation in a price-sensitive market place. All data presented in this study are expressed in percentage of meat proportions and not DNA contents.

### *Calculations*

For the calculation of proportions, the DNA contents measured of all species are summarized and normalized to 100%. Proportions are calculated for each species individually. These measured proportions in percent were the raw data that were collected during this ring trial.

## Results

Nineteen laboratories from Switzerland, Germany, and Austria produced 21 datasets. One dataset was excluded from evaluation because its data did not include results for horse. Two other laboratories produced two datasets, each applying two different PCR methods.

In total, 3808 data points were collected (not presented). All participants used the standard reference sausages (KLJ and KBW), as well as their own DNA dilution series to calibrate their assays. The lowest proportion of meat in the calibration sausages consisted of 1% for each species, and all datasets generated positive results at this level. We conclude, therefore, that the detection limit is at least 1% of meat content for all four species. No data were assigned as outliers and excluded.

**Table 5. Data from all valid data sets from all laboratories[a]**

| Sample | Parameter[b] | Cevapcici | | | | Landjäger | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Beef | Pork | Sheep | Horse | Beef | Pork | Sheep | Horse |
| KDNA | Measured mean value | 52.8 | 15.1 | 24.0 | 8.1 | 35.5 | 36.8 | 2.9 | 24.8 |
| | True value | 50 | 15 | 25 | 10 | 25 | 43 | 2 | 30 |
| | r | 14.4 | 10.2 | 14.8 | 4.0 | 9.1 | 10.6 | 2.0 | 7.5 |
| | R, % | 27.2 | 25.0 | 30.0 | 6.9 | 20.5 | 29.8 | 3.3 | 22.7 |
| | Deviation, % | 6 | 0 | –4 | –19 | 42 | –14 | 45 | –17 |
| | MU (ext) rel., % | 38 | 117 | 89 | 76 | 72 | 66 | 102 | 77 |
| KLJ | Measured mean value | 43.1 | 18.5 | 24.1 | 14.3 | 25.7 | 36.0 | 2.4 | 35.9 |
| | True value | 50 | 15 | 25 | 10 | 25 | 43 | 2 | 30 |
| | r | 12.7 | 13.0 | 14.0 | 6.6 | 6.9 | 12.5 | 2.3 | 11.8 |
| | R, % | 21.5 | 20.6 | 17.6 | 9.5 | 11.3 | 13.9 | 3.0 | 16.2 |
| | Deviation, % | –14 | 23 | –4 | 43 | 3 | –16 | 20 | 20 |
| | MU (ext) rel., % | 48 | 87 | 52 | 76 | 31 | 47 | 95 | 46 |
| KBW | Measured mean value | 50.7 | 16.4 | 24.2 | 8.6 | 31.5 | 39.8 | 2.5 | 26.2 |
| | True value | 50 | 15 | 25 | 10 | 25 | 43 | 2 | 30 |
| | r | 8.9 | 9.9 | 12.1 | 4.0 | 7.2 | 7.7 | 1.8 | 7.9 |
| | R, % | 23.7 | 14.2 | 16.7 | 5.7 | 15.6 | 16.8 | 2.5 | 17.6 |
| | Deviation, % | 1 | 10 | –3 | –14 | 26 | –8 | 25 | –13 |
| | MU (ext) rel., % | 33 | 63 | 49 | 56 | 54 | 34 | 81 | 55 |

| | | Salami | | | | Sukuk | | | |
|---|---|---|---|---|---|---|---|---|---|
| KDNA | Measured mean value | 9.5 | 44.9 | 7.0 | 38.6 | 53.4 | 2.9 | 39.7 | 4.1 |
| | True value | 5.0 | 48.0 | 2.0 | 45.0 | 55.0 | 4.0 | 37.0 | 4.0 |
| | r | 4.1 | 12.2 | 6.1 | 14.6 | 14.4 | 4.3 | 16.5 | 2.3 |
| | R, % | 7.4 | 35.7 | 9.1 | 33.8 | 33.4 | 6.7 | 35.6 | 3.5 |
| | Deviation, % | 90 | –7 | 249 | –14 | –3 | –27 | 7 | 2 |
| | MU (ext) rel., % | 110 | 58 | 170 | 70 | 45 | 179 | 65 | 61 |
| KLJ | Measured mean value | 6.6 | 37.0 | 5.3 | 51.1 | 46.9 | 4.3 | 41.0 | 7.8 |
| | True value | 5.0 | 48.0 | 2.0 | 45.0 | 55.0 | 4.0 | 37.0 | 4.0 |
| | r | 2.6 | 23.3 | 5.8 | 11.8 | 17.1 | 5.5 | 21.4 | 7.1 |
| | R, % | 4.3 | 27.4 | 7.2 | 22.0 | 20.8 | 6.6 | 22.7 | 8.5 |
| | Deviation, % | 33 | –23 | 166 | 14 | –15 | 7 | 11 | 96 |
| | MU (ext) rel., % | 68 | 79 | 157 | 39 | 47 | 110 | 44 | 124 |
| KBW | Measured mean value | 7.8 | 45.2 | 6.3 | 40.6 | 52.8 | 3.2 | 39.7 | 4.3 |
| | True value | 5.0 | 48.0 | 2.0 | 45.0 | 55.0 | 4.0 | 37.0 | 4.0 |
| | r | 3.1 | 9.6 | 6.0 | 11.0 | 10.3 | 5.1 | 11.8 | 2.7 |
| | R, % | 6.6 | 20.5 | 7.9 | 22.8 | 20.0 | 6.2 | 19.8 | 3.0 |
| | Deviation, % | 57 | –6 | 216 | –10 | –4 | –20 | 7 | 8 |
| | MU (ext) rel., % | 94 | 34 | 163 | 45 | 28 | 146 | 38 | 51 |

[a] Four unknown samples were measured using matrix adapted standards (KLJ and KBW) or dilutions of genomic DNA from muscle tissue (KDNA). Each sample was extracted twice, and each extract was analyzed twice. Results are % (w/w).

[b] r = Repeatability; R= comparability; Deviation = relative deviation from the true value; MU = extended measurement uncertainty with an extension factor of 2 within a 95% interval. This means that the single measured value corresponds to the true value in the given interval MU (ext) rel. (e.g., Sukuk, KBW for beef: ±28%) with a probability of 95%.

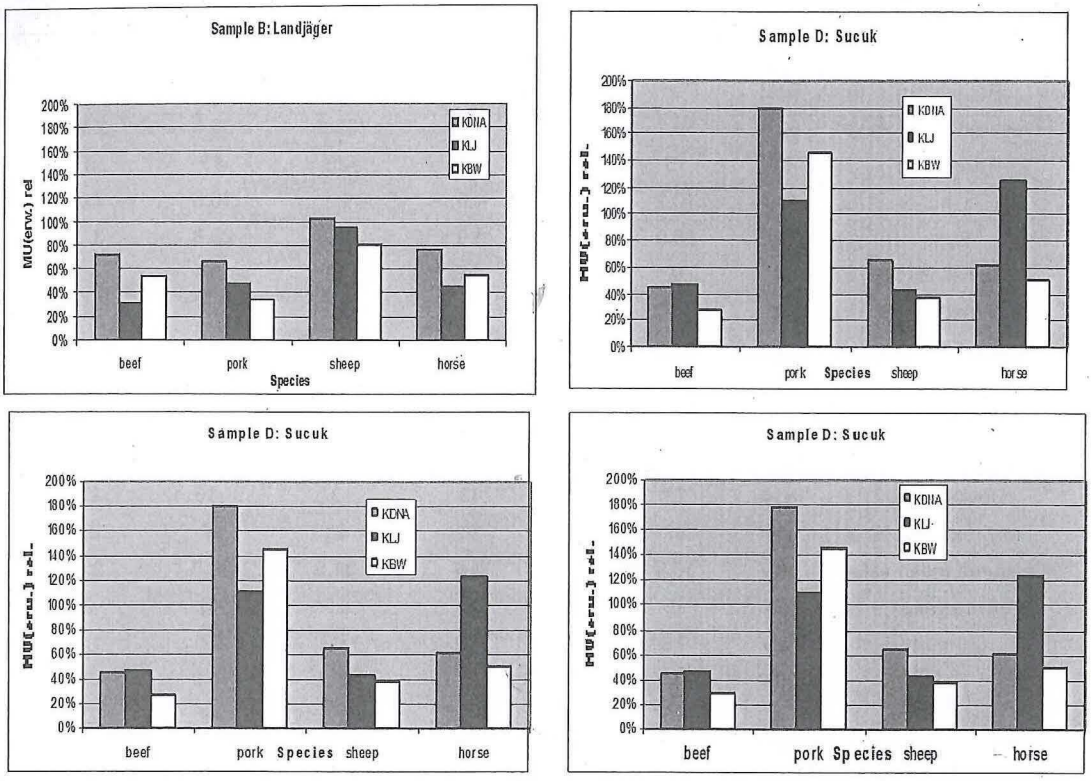


**Figure 1. Graphic presentation of extended MU after calibration with KDNA or matrix-adapted reference sausages (KLJ, KBW), based upon data presented in Table 5. In general, values generated using calibration with matrix-adapted reference sausages (KBW) of fine texture exhibited lower measurement uncertainties. Minor components exhibited higher measurement uncertainties (see sample salami, species sheep, 2% and sample sukuk, species pork, 4%). Every laboratory used its own DNA dilution series composed of all four species.**

*z*-Scores were calculated for each data set (for calculation and results *see* Table 4) to estimate the relative performance of the different methods associated with various laboratories. There was no obvious difference between the *z*-scores of results derived from a different thermocycler, sample size, and DNA isolation method. The highest z-score was assigned to the use of a different PCR system.

### *Calibration by Dilutions of DNA*

When genomic DNA dilutions from muscle tissue (KDNA) were used for calibration, an average deviation of 34% from the true values was calculated over all datasets. Table 5 shows values using KDNA for calibration; Figure 1 shows a graphical representation. The measurement uncertainty (MU) averaged 87% over all datasets and all species. This calibration strategy does not take into account that different types of tissue, like fat, muscle, connective tissue, and skin, may exhibit different DNA concentrations.

### *Calibration by Matrix-Adapted Reference Sausages, Type LJ*

When this type of reference sausages was used for calibration, an average deviation of ±32% from the true values was calculated over all datasets (Table 5). Figure 1 shows a graphical representation. The MU averaged 72% over all datasets and all species. Minor components of lower than 5% exhibited a high measuring uncertainty of 95% or more (*see* graphical representation of MU in Figure 1). A higher MU for contaminating components must be expected, as such components enclose all criteria for higher MU like inhomogenity due to large particles. But in practice precise quantification in this range is often less important, because such minor components are often a result of unconscious cross-contamination, reflecting bad production practice, not fraud.

### *Calibration by Matrix-Adapted Reference Sausages, Type BW*

When this type of reference sausages was used for calibration, an average deviation of ±27% from the true values was calculated over all datasets (Table 5); a graphical representation is shown in Figure 1. The MU averaged 64% over all datasets and all species. Minor components of lower than 5% exhibited a high MU of 51% or more (*see* graphical representation in Figure 1). Calibration by matrix-adapted reference sausages led to the lowest measurement uncertainty.

## Discussion

An earlier publication (14) described an interlaboratory trial showing that the measurement of proportions of beef, pork, turkey, and chicken in sausages by measuring DNA contents may be feasible. Results with an acceptable MU could be produced in the case where matrix-adapted reference material was applied as calibrator. The impact of the DNA isolation method, thermocycler, and the applied PCR-system was not significant.

In this study we showed that this may be also possible for meat products containing horse, beef, sheep, and pork. Obvious differences in MUs, influenced by the DNA isolation method or thermocycler used, were again not found, confirming the earlier findings (14, 17).

Accordingly, differences between calibration methods were expected and evaluated. Surprisingly, processing like ripening or boiling (except the homogeneity of the texture) did not significantly influence the outcome of the measurement. This is a very important finding. It may favor the predominant production of boiled, storable, fine-textured reference sausages, which may also be used as calibrators for raw products. This reduces the number of required calibrators.

In addition, the species composition of the samples seems to have no obvious impact on the MU. All tested combinations showed acceptable MUs. However, use of matrix-adapted standards of fine texture (KBW) resulted in lowest MU. A possible explanation for these findings, contrasting to earlier findings, may be that all species are closely related mammalian with similar nature, consistency and edible cuts of meat. Therefore, DNA contents of the meat of these species may be more similar than those between poultry and mammalian used in the earlier study (14).

On the basis of these results it can be summarized, that the measurement of DNA contents with multiplex real-time PCR in conjunction with matrix-adapted reference sausages enables laboratories to reproducibly determine meat proportions according to the applied recipes.

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