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ORIGINAL PAPER

Quantification of beef and pork fraction in sausages by real-time PCR analysis: results of an interlaboratory trial

Albert Eugster · Jürg Ruf · Jürg Rentsch · Philipp Hübner · René Köppel

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Abstract The performance of quantitative PCR-methods for the determination of beef and pork fraction in sausage was tested in an interlaboratory trial. Twelve different laboratories analysed four sausages of different composition of beef and pork by using four sausages of known fraction of meat as calibrators. Although different PCR-methods were applied, the precision of all results was better than 16% and the trueness better than 25%. The main reason for the good performance is the use of common calibrators emphasizing the importance of the quality of calibrators for molecular analysis. Thus, we conclude, that PCR-based detection methods are suitable for quantitative control of meat fractions in sausages.

Keywords Species identification · DNA quantification · Quantitative real-time PCR · Kalbsbratwurst · Sausage · Interlaboratory trial · Veal · Beef · Pork

A. Eugster
Cantonal Office of Consumer Protection Aargau,
Aarau, Switzerland

J. Ruf
Official Food Control Authority of the Canton Thurgau,
Frauenfeld, Switzerland

J. Rentsch
Swiss Quality Testing Services (SQTS),
Courtepin, Switzerland

P. Hübner
Public Laboratory of the Canton Basel-Stadt,
Basel, Switzerland

R. Köppel (✉)
Official Food Control Authority of the Canton of Zurich,
Zurich, Switzerland
e-mail: rene.koeppel@klzh.ch

Introduction

Fried sausages of veal (“Kalbsbratwurst”) are consumed in bulk quantities in Switzerland and its neighbouring countries. By law “Kalbsbratwurst” must be composed of at least 50/100 g veal and less pork [1]. As veal is much more expensive than pork, fraud may be attractive for producers in a highly competitive market. The determination of meat fractions in processed food such as sausages is therefore an important issue for official control laboratories. Usually, fraud encloses sausages with diminished content of veal. Only seldom the sausages contain no veal at all. To prosecute producers for their fraud, analytical methods must be able to quantify all expected components in this complex matrix.

Precision and accuracy are crucial performance criteria to ensure comparable results between control laboratories. Although PCR-based methods cannot distinguish veal from beef, they proved their applicability for the analysis of mixed processed food already in the past [8]. The precision and accuracy of PCR-methods for food species was usually estimated by comparing results generated by the same method [2–7, 9–11] and relative standard deviations (RSD) of about 30% were reported.

A basic problem of DNA-based methods influencing their accuracy is the composition of the sample. Sausages contain different tissue material, ranging from fatty bacon to fatless meat and connective tissue. These different tissues exhibit different concentrations of DNA and therefore the initial weight of meat in sausages may not be reflected by the species-specific DNA proportion received after DNA-isolation. This may lead to biased results generated by DNA-based detection methods. One approach often applied to compensate this possible lack of accuracy is to use appropriate reference material as calibrators simulating the

real production process. In this study, four different reference sausages were produced as calibrators containing 30–60/100 g veal fraction in pork according to a traditional recipe of "Kalbsbratwurst". Twelve laboratories from Switzerland and Germany used their own DNA extraction and PCR methods to determine the fractions of veal in four unknown samples using the above mentioned reference sausages for quantification. Here we present the results of this approach.

Materials and methods

Reference sausages

Reference sausages were produced according to the composition described in Table 1. According to a traditional recipe of "Kalbsbratwurst", 10 kg of each sausage mixture was produced by the Master Butcher School Spiez, Switzerland. Reference sausages were analysed on the content of water, fat, total protein, connective tissue protein and muscle protein. The principal components were consistent with an average, commercially obtainable "Kalbsbratwurst" as estimated in a market analysis from 1965 to 1997 [12] (data not shown).

Samples A–D

Sample sausages A and B were produced similarly to the reference sausages by the Master Butcher School Spiez, Switzerland. Sample C was a sausage from the market. Sample D is a local speciality with a slightly different composition, mainly by addition of milk soaked white bread. The overall fat content is similar to the other sample sausages but with a higher relative fat proportion originating from veal lumbar fat. Thus, the composition of the unknown sausages differed significantly. An overview on the detailed composition of the samples is given in Table 2.

Table 1 Composition of reference fried sausages ranging from 60/100 to 30/100 g veal proportion

Portion of veal	60/100 g	50/100 g	40/100 g	30/100 g
Reference sausage composition				
Veal	38.5	31.7	24.9	18
Pork	2.9	9.8	16.6	23.4
Bacon	24.4	24.4	24.4	24.4
Calf's head incl. 50% ice	4.9	4.9	4.9	4.9
Ice/water	29.3	29.3	29.3	29.3
Total	100	100	100	100

Table 2 Composition of sample fried sausages

Portion of veal[a]	A (34/100 g)	B (48/100 g)	D (54.8/100 g)
Sample fried sausage composition			
Veal	20.8	30.3	50 fatty
Pork	20.7	11.1	26.2 fatty
Bacon	24.4	24.4	19
Calf's head incl. 50% ice	4.9	4.9	4.8 [b]
Ice/water	29.3	29.3	Unknown
Total	100	100	100

The composition of sample C is unknown; the estimated value for the veal portion is 50 g/100 g

[a] Sum of veal meat proportion to the sum of total meat, calculated from the recipe

[b] With no ice

DNA based methods

A compilation of DNA-isolation and real-time PCR methods used is shown in Table 3.

Results

Eleven datasets of a total of 14 datasets produced by all 12 laboratories were used for the statistical evaluation. Three datasets did not correspond to the minimal requirements as previously agreed upon, and were excluded. The included data sets are presented in Table 4. All participants used the standard reference sausages to calibrate their assays. The statistical analysis of the datasets revealed that all mean values were very close to the true values of the recipe. The largest bias was observed for sample D with −5.6/100 g. Real-time PCR based detection methods often display a relative standard deviation (RSD_R) of 30% [13]. In this study, different PCR-methods were performed in participating laboratories. Therefore, we expected an elevated value for the RSD, taking into account that different PCR-systems may differ in their performance caracteristics. In contrary to this expectation, a maximal RSD_R value of 18% was found (sample C; mean value 48.8/100 g, reproducibility s_R 8.9/100 g) leading to an extended uncertainty of measurement of 18/100 g (Table 5). Not unexpectedly, results of samples A and B, which were produced similarly to the reference sausages used as calibrators, exhibited the smallest values for repeatability (s_r 1.4/100 and 3.5/100 g) and for reproducibility (s_R 2/100 and 3.6/100 g) resulting in a minimal extended uncertainty of measurement of 4.3/100 g. It is worth mentioning that we found no significant difference between results generated with or without sample homogenization prior to DNA extraction, and no differences dependent on sample size, DNA extraction method and thermocycler used.

Table 3 Compilation of the methods used by the participating laboratories 1–11

Laboratory	1 and 3	2	4	5	6	7	8	9	10	11
Sample size (mg)	300	300	300	45	300	300	2000	100	300	300
Homogenization yes or no	Yes	Yes	No	No	Yes	Yes	Yes	No	Yes	No
DNA Isolation	Wizard	Wizard	Wizard	QiAmp	Wizard	Wizard	CTAB	CTAB	CTAB	CTAB+Qiagen
Replicates of DNA-isolation	4	4	4	4	4	1	4	4	4	4
Type of Real-time PCR	simplex	simplex	simplex	simplex	duplex	simplex	simplex	simplex	simplex	simplex
PCR-system for beef	PDE	PDE	beta-Actin	PDE	LIF	PDE	beta-Actin	PDE	Laube et all	Laube et all
PCR-system for pork	ryanodin	ryanodin	beta-Actin	ryanodin	ryanodin	PGH	beta-Actin	ryanodin	Laube et all	Laube et all
Nr. of replicates per PCR result	2	2	2	2	2	0	2	0	2	2
Thermocycler	ABI 7900	RG 3000	RG 3000	ABI 7300	ABI 7900	ABI 7700	ABI 7500	ABI 7900	ABI 7900	Light cycler

For the DNA isolation four different methods, for PCR-amplification three methods for each species and seven different models of thermocyclers were applied. Only one Laboratory used duplex PCR-amplification

Table 4 Values of four fried sausages (sample A–D) measured by 11 of 12 participating laboratories applying their different methods

Laboratory	1	2	3	4	5	6	7	8	9	10	11
Sample A											
1. DNA isolate	31	35	33	35	30	35	33	36	34	36	32
1. DNA isolate	32	31	23	33	31	34	34	35	35	35	29
2. DNA isolate	32	33	34	36	30	34	35	32	35	34	32
2. DNA isolate	33	33	28	37	32	34	32	35	35	34	32
Sample B											
1. DNA isolate	51	45	22	52	44	48	42	47	57	47	50
1. DNA isolate	46	50	31	42	45	48	28	48	57	47	47
2. DNA isolate	52	44	44	51	43	48	55	49	47	45	49
2. DNA isolate	50	45	48	52	50	48	47	45	45	46	46
Sample C											
1. DNA isolate	52	46	47	44	52	39	36	53	63	55	59
1. DNA isolate	52	41	48	42	47	35	37	50	65	56	58
2. DNA isolate	54	46	51	44	44	38	37	49	67	53	57
2. DNA isolate	55	44	48	43	44	37	35	47	68	53	58
Sample D											
1. DNA isolate	55	51	47	51	44	43	51	49	56	48	52
1. DNA isolate	55	47	38	46	45	43	56	49	56	47	51
2. DNA isolate	56	52	51	52	58	44	36	52	48	48	52
2. DNA isolate	52	46	44	48	59	43	33	51	61	47	56

Each sample was extracted twice and each extract was analysed twice. Results are in percent beef

[a] Promega, Madison, WI, USA

[b] see lit. 12 (SLMB)

[c] Qiagen, Hilden, Germany

Discussion

Our study clearly demonstrates that the reliable, accurate and precise measurement of the beef fraction of sausages is feasible independent of the applied PCR-based detection method. As expected, repeatability and reproducibility values are better for samples closely resembling the reference sausages used as calibrators. Of course, it is not realistic to produce reference sausages for every single type of sausage on the market. However, it can be done for those types of products where legal compositional requirements have to be enforced.

Further studies will be needed to validate the application range of a given set of reference sausages. Such studies will help to define a minimal set of reference sausages for the DNA-based quantitative detection of meat fractions. Analytical limitations might be expected outside the range covered by the actual set of reference sausages. But it is also of particular interest to quantify minor amounts of non-

Table 5 Comprehensive statistical calculations of the included eleven datasets

	Sample A	Sample B	Sample C	Sample D
Beef true value (g) [a]	34/100	48/100	[b]50/100	54.8/100
Mean value (g)	33.3/100	47.8/100	48.8/100	49.3/100
Bias (g)	−0.7/100	−0.2/100	−1.2/100	−5.6/100
Repeatability S_R (g)	1.4/100	3.5/100	2.0/100	5.0/100
Reproducibility $S_{R\,(g)}$	2.0/100	3.6/100	8.0/100	5.9/100
RSD_R (%)	6.0	7.5	18	12.0
Uncertainty [c] (95%, g) ±	4.3/100	7.2/100	18/100	16.3/100

The maximal expanded uncertainty of measurement was determined as 18/100 g for sample C

[a] calculated from the recipe

[b] The exact recipe is not known. The estimation made by experience was 50%

[c] Expanded uncertainty of measurement

declared meat additions in terms of a good manufacturing praxis (GMP) or the meat fraction of improper species in certain ethnic specialities.

We resume that the presented approach is feasible and gives accurate and precise results independent of the applied PCR-methods.

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