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THE APPLICATION OF A EURL-AP RECOMMENDED PROTOCOL FOR DETECTION OF RUMINANT DNA IN FEED AND MEAT*

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The European Commission recommends a EURL-AP (European Union Reference Laboratory for Animal Proteins in Feedingstuffs) protocol to identify ruminant DNA. The aim of the study was to test method performance and to determine its parameters during internal and external validation. The experiment used ruminant specific DNA plasmid (ERM-AD482), reference feed samples containing no animal components, and feed samples containing bovine, ovine and porcine meal (0.2% each), poultry meal (100%), poultry plasma, pork plasma, beef samples, and pork samples. In the first stage of the study, the DNA plasmid was used to adjust the method to the StepOnePlus Real-Time system, in which the analysis was performed. This was aimed to determine the cut-off line (c_T), or the threshold limit value distinguishing between positive and negative results. Next, DNA was extracted from standard samples using a Wizard Food DNA Isolation Kit recommended by the EURL-AP. The obtained DNA was subjected to Real-Time PCR using primers compatible with the sequence flanking the species specific region for ruminants as well as a probe compatible with ruminant DNA. All the samples were analysed in two repetitions of DNA isolation. Each DNA isolate was additionally analysed at a 10-fold dilution. Amplification threshold values (c_T) were determined for all the matrices. The calibration result shows that the cycle threshold value between positive and negative samples is c_T =33.8. This served as a basis for determining the samples with ruminant DNA. Positive reactions were only obtained for ruminant DNA (bov 0.2%, ovis 0.2%); for the other species, the reaction always occurred beyond the 34th cycle. For the positive samples, in the case of DNA dilutions, the threshold cut-off value moves every time by around 3 cycles. This method is highly sensitive, identifying processed material in amounts of <0.1%. Our study showed this method to be 100% species specific.

Key words: DNA identification of ruminants, EURL-AP

An effective prohibition of using processed animal proteins (PAP) is the only way to prevent the spread of Transmissible Spongiform Encephalopathies (TSE), one

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of which is BSE disease. Establishment of strict regulations concerning this issue led to a complete prohibition of using processed animal proteins in feed, which has been effective in Europe for many years. What is more, species identification of the processed food components is of remarkable interest of the consumers and food manufacturers. A general need to get to know the actual composition of food products results from the increasing care of product quality associated with an increase in social awareness concerning the effect of food on human health. Laboratory methods whose application is also regulated by law are needed in order to reliably provide the composition of a food product or effectively enforce standards concerning feed or food.

For several dozen years, classical optical microscopy has been an official standard method of detection of processed animal protein in feed mixtures in the European Union (Fumière et al., 2009). Due to its limitations, associated among others with lack of a possibility to recognize the species in the present meals, the European Commission approved, in orders 152/2009 and 51/2013, a method of analysis based on DNA analysis that would provide more information on the origin of animal protein.

The aim of the present study was to validate the method proposed by EURL-AP of identification of animal-derived components originating from ruminants in feed and meat.

Material and methods

Study material consisted of ruminant specific DNA plasmid (ERM-AD482), reference feed samples containing no animal components, and plant-based feed samples containing bovine, ovine and porcine meal (0.2% each). The plasmid was obtained from the Institute for Reference Material at the European Commission (ERM-European Reference Materials), whereas reference feed samples – from proficiency testing (AHVLA). Moreover, our own reference materials were used: poultry meal (100%), poultry plasma, pork plasma, beef samples and pork samples.

Experimental methods

In the first stage of the study, with the use of plasmid, the method was adjusted to Real-Time device, on which the analysis was to be conducted (StepOne Plus – Thermofisher). Then, DNA was extracted from standard samples using a Wizard Food DNA Isolation Kit recommended by the EURL-AP. The obtained DNA was subjected to RealTime PCR reaction with the use of primers flanking the species-specific region for ruminants and a probe compatible with ruminant DNA. All the samples were analysed in two repetitions of DNA isolation. Each DNA isolate was additionally analysed at a 10-fold dilution. All the isolations were performed in the presence of positive controls (KP) and negative controls (KN) of isolation; a positive control (PTC) and a negative control qPCRu (NTC) were added to each series of reactions. Commercial DNA samples of the individual species with composition declared by the manufacturer or samples from the proficiency testings were used as PTC control.

Amplification threshold values (c_T) were determined for all the matrices. Results c_T were presented for the same sample from two measurements, and relative standard

deviation between these measurements. The number of cut-off copies was established based on amplification threshold value.

On the basis of threshold values, the samples containing ruminant DNA were determined. Parameters of the method, such as specificity, accuracy, limit of detection and sensitivity were calculated using the following definitions: – specificity of the method as part of the actually negative results among all the negative samples that were analysed;

– accuracy of the method as part of the number of properly determined results to all the obtained results expressed by the formula; – limit of detection (LOD) as the lowest concentration or the amount of product that is possible to detect with the use of certain analytical procedure and sensitivity of the method as proportion of actually positive results in all the positive samples subjected for analysis.

Results

The calibration result shows that the cycle threshold value between positive and negative samples is c_T =33.8. On the basis of this value, the samples containing ruminant DNA were determined. Results, in the form of amplification threshold values, relative standard deviation and assessment of presence of ruminant DNA in a sample, are presented in table 1. A number of cut-off copies was 14.77.

Table 1. Results of ruminant DNA determination

No. of sample	c_T	RSD%	Result	No. of sample	c_T	RSD%	Result
1	2	3	4	5	6	7	8
Bov 0,2%	22.78	0.75	+	Mięso bov 100%	15.98	0.68	+
Bov 0,2%	22.54		+	Mięso bov 100%	15.83		+
Bov 0,2% 1:10	25.37	0.56	+	Mięso bov 100%	18.00	0.29	+
Bov 0,2% 1:10	25.58		+	Mięso bov 100%	17.93		+
1 Bov 0,2%	22.49	0.44	+	Mięso sus 100%	34.52	1.54	-
1 Bov 0,2%	22.36		+	Mięso sus 100%	35.28		-
1 Bov 0,2% 1:10	24.85	0.30	+	Mięso sus 100%	33.91	5.88	-
1 Bov 0,2% 1:10	24.75		+	Mięso sus 100%	35.77		-
Sus 0,2%	36.69	0.61	-	Plazma sus	33.96	5.27	-
Sus 0,2%	36.38		-	Plazma sus	35.84		-
Sus 0,2% 1:10	38.37	1.22	-	Plazma sus 1:10	35.94	2.80	-
Sus 0,2% 1:10	39.04		-	Plazma sus 1:10	37.34		-
Gall 100%	37.88	1.02	-	Plazma drob	35.34	10.97	-

Table 1 contd.

1	2	3	4	5	6	7	8
Gall 100%	37.33		-	Plazma drob	41.29		-
Gall 100% 1:10	39.45	1.24	-	Plazma drob 1:10	34.46	7.45	-
Gall 100% 1:10	40.15		-	Plazma drob 1:10	38.29		-
Ovis 0,2%	26.034	0.06	+	RM neg	37.97	0.43	-
Ovis 0,2%	26.06		+	RM neg	38.21		-
Ovis 0,2% 1:10	28.88	0.42	+	RM neg 1:10	38.43	0.02	-
Ovis 0,2% 1:10	28.71		+	RM neg 1:10	38.45	0.75	-
KN izol	35.41	0.48	-	NTC	43.04	0.12	-
KN izol	35.17		-	NTC	42.96		-

Amplification threshold value (c_T), relative standard deviation (RSD%) and the result, namely the interpretation if a given sample contains ruminant DNA are presented in Table 1.

Notes: Mieszanka paszowa Ovis 0.2% – feed mixture containing 0.2% ovine meal; mieszanka paszowa Bov 0.2% and mieszanka paszowa 1 bov 0.2% – feed mixtures each containing 0.2% bovine meal; mięso bov 100% – beef meat; mięso sus 100% – pork meat; plazma drob – plasma from poultry blood; plazma sus – plasma from porcine blood.

Discussion of the results

In all the samples that do not contain ruminant DNA in their ingredients the c_T values were above the threshold value, whereas ruminant DNA had lower values. Based on the obtained c_T threshold values it can be concluded that the method is species specific (table 1) (specificity, sensitivity and accuracy amount to 100%). Positive reactions were obtained only for ruminant DNA (bov 0.2%, ovis 0.2%), whereas in case of DNA of other species, the reaction always occurred after the 34 cycles. The presented results concerning the c_T value unambiguously indicate he repeatability of the results for the positive samples – differences in c_T values were slight, and relative standard deviation was lower than or equal to one cycle. In case of non-ruminant DNA, the repeatability of the results was varied and amounted to from approximately 1.5 to 6 cycles.

In addition, it is worth noting that for positive samples, in case of dilution, the threshold cut-off shifts by approximately 3 cycles, whereas in case of negative samples, threshold sometimes remains constant.

The method is very sensitive; it enables to identify the processed material in 10-fold dilution, namely in the amount of <0.02%. Such value was accepted for LOD of the method.

Methods based on PCR or its modification in real time (Real-Time PCR) play the main role in species identification. It should be noted that despite the fact that

DNA is a stable molecule, that can withstand hyperthermia and hyperbaric processing during the manufacturing of animal meals, DNA chain is fragmented to pieces of size of approx. 100 bp. Due to this reason, the best effects of analysis are obtained using DNA fragments that are slightly shorter (Fumière et al., 2006; de la Torre et al., 2007). Real-Time PCR technique is used more often in order to identify such short fragments, due to higher sensitivity of this method and better accuracy thanks to the use of probes. The obtained results explicitly confirm the effectiveness of this method. Both the samples with considerable hardness (e.g. meat and bone meals) and the by-products not containing bones (dried plasma, meat) can be analysed with the use of this method.

It is worth noting that the development of analytical methods serving for detection and species identification of processed animal proteins (PAP) in feed on the basis of mtDNA analysis is very important in animal breeding, because it gives a chance to possibly terminate the prohibition of cross-feeding the non-ruminant animals that are not intended for consumption. However, a possibility of verification of species content of food contributes to authentication of actual content of the product.

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SUMMARY

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