ELSEVIER

Meat Science 54 (2000) 1–7

MEAT SCIENCE

www.elsevier.com/locate/meatsci

Determination of an appropriate heat treatment of animal waste using the ELISA technique: results of a validation study

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Received 9 February 1999; accepted 3 July 1999

Abstract

Effective sterilisation of animal waste is an important prerequisite for the use of animal meal as an ingredient of compound feed for non-ruminants. Specific conditions for the rendering process aimed at the inactivation of the causative agent of Bovine Spongiform Encephalopathy (BSE), and the prevention of other pathogens in feedingstuff, are defined by European law. A validation study encompassing 21 laboratories from 12 European countries was performed using a commercially available test kit based on an enzymelinked immuno sorbent assay (ELISA) which allows proof of the appropriate heat treatment of the processed animal waste. The evaluation of the results regarding the *R*-value reveals the robustness of the method, a sufficient sensitivity to detect a deviation of the processing conditions from the regulation and a low standard deviation of the data. Moreover the statistical evaluation of the results allows for the estimation of the coefficient of variation which is between 11 and 22% depending on the magnitude of the response of the test.

1. Introduction

Animal meal, due to its high nutritional value, is an important ingredient of compound feed for non-ruminants like poultry and pigs. In order to avoid a further spread of BSE, and other epidemic diseases, it has to be assured that the production of animal meal fulfils certain criteria laid down in Decision 96/449/EC (European Commission, 1996). It is strongly assumed that the causative agent of BSE is inactivated when the animal material is treated for 20 min at 133°C (406 K) and 3 bar under wet sterilisation conditions. While the technical control of a rendering plant using temperatures and pressure measures is mandatory within the European Union there is an urgent need for a method that can be used for the surveillance of the processed material. A commercially available ELISA test kit for the identification of pork in cooked meat has been used in this study. The general applicability of this kit to the specific purpose of the control of the proper heat treatment of animal meal is based on the fact that animal meal is a mixture produced from animal species like cows, pigs, poultry and sheep. Therefore, the result of the analysis of an animal meal sample using the ELISA kit would be expected to be positive. However, it was demonstrated that the response of this test depends strongly on the temperature of the heat treatment of the pork (Hofmann, Fischer, Mueller & Kemper, 1995), which is indicated by a significant loss of sensitivity of the test if the heat treatment took place at 133°C (406 K). These results led to the examination of animal meal samples from rendering plants (Hofmann, 1996) which revealed the applicability of the ELISA test to check a proper heat treatment of animal meal. In 1997, a ringtest has been already carried out in Germany, confirming the robustness of the method (Hofmann, 1998). Moreover this technique is today already applied in routine analysis to the surveillance of some rendering plants (Unglaub, 1997; Unglaub, Mueller, Jemmi & Stuker, 1998).

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0309-1740/99/$ - see front matter
PII: S0309-1740(99)00087-X

Animal meal is produced in the European Union employing different techniques. However, all of them have to comply with Decision 96/449 EC (European Commission, 1996) in order to ensure a sufficient sterilisation of the processed material. The ELISA test kit allows for an examination of the product itself but information concerning the precision of the method for this specific purpose is still missing. Moreover, the estimated uncertainty should reflect the variability of a method that is employed in different Member States of the European Union. The knowledge of basic quality criteria such as the coefficient of variation (CV) or lack of bias of the method are required in order to determine a reliable tolerance limit that can be used to detect non proper heat treatment.

The aim of this study was to evaluate the precision of the ELISA method by conducting a collaborative trial encompassing 21 laboratories from 12 countries of the European Union and Switzerland. The coefficient of variation of this method reflecting the variability of the data and the average value of the results of all laboratories should be estimated. The average value could be used to examine the lack of bias of the method. The general outline of the study, the method applied and the statistical analysis of the collaborative trial are presented in this paper.

2. Materials and methods

2.1. Test material

For this validation study, four different materials from two animal meal plants in Germany (Zweckverband Tierkoerperbeseitigung Warthausen and Zweckverband Protec Orsingen, both Baden-Wuerttemberg, Germany) and a meat and bone meal plant from Switzerland (Ed. Geistlich Soehne AG) were used.

While the composition of the test material represents typical processed mammalian animal waste such as animal meal and meat-and-bone meal, the individual types of the material were treated in different ways to ascertain a broad range of different responses of the ELISA method in the study (Table 1).

Prior to the use of the processed animal waste as test material the homogeneity was investigated. The control of a sufficient homogeneity of the test material being filled into bottles is an important prerequisite for an unambiguous interpretation of the results of the validation study. For reasons of clarification it should be pointed out that in this case the homogeneity of the material does not reflect an equal distribution of a certain compound in the material but the equal heat treatment of every part of the bulk material. A part of the material is defined as the amount of the sample subjected to the ELISA-analysis.

2.1.1. Preparation of the material

The preparation of the test material was carried out by the Institute of Reference Materials and Measurement of the European Commission in Geel, Belgium and comprised the following steps. At first, each sample of material was sieved through a 1 mm sieve in order to remove foreign matter such as rubber and metal pieces. The sieving step does not change the composition of the test material significantly since the animal meal has been previously ground during the processing of the animal waste. Subsequently the material was thoroughly mixed for 3 h in a Turbula mixer and finally filled into 60 ml brown glass bottles. Each bottle contained 20 g test material.

2.1.2. Test for homogeneity

In order to ensure a high homogeneity of the animal meal the test for homogeneity was carried out in *two* steps. In the *first* step samples were taken from the bulk material and analysed prior to the packaging of the animal meals. The relative standard deviation of the analysis using the ELISA method ranged from 6 to 12% indicating a good homogeneity of the raw material. In the next step, the test material was prepared for bottling as described in the preceding paragraph.

Finally the homogeneity of the test material filled into the bottles was investigated as described by Thompson and Wood (1993): 10 sample glasses were randomly selected and two samples of about 6 g from each glass were taken and subjected to the ELISA-analysis thus performing 20 analysis from each material. The statis-

Table 1
Characterisation of the test material

Material	Treatment
Animal meal I (AM I)	20 min, 133°C (406 K)–135°C (408 K), (3–3.2 bar)[a], wet sterilisation
Animal meal II (AM II)	20 min, 128°C (401 K)–130°C (403 K), (2.6–2.8 bar)[a], wet sterilisation
Animal meal III (AM III)	20 min, $T < 128$°C (401 K) , $P < 2.6$ bar[a], wet sterilisation
Meat and bone meal (material prior to the subsequent sterilisation)	Dry sterilisation 90°C (363 K)- → 140°C (413 K), 105 min; for 20 min $T > 133$°C (406 K) , without overpressure

[a] The pressure was calculated according to the employed temperature.

tical method "analysis of variance" (ANOVA) was employed in order to prove a significant difference of the variability *between* the glasses and the variability *within* the glasses. It is assumed that the variance between the glasses represents the heterogeneity of the material while the within sample variance is attributed to the analytical error of the ELISA method. The results shown in Table 2 confirm a sufficient homogeneity of all materials as the respective *F*-values did not exceed the critical *F*-value ($F = 3.02$) for a confidence level of 95% ($\alpha = 0.05$) thus indicating that the variability between the bottles is **not** significantly higher than the analytical error.

2.2. Methods

2.2.1. Outline

Since the main aim of this study was to determine the precision of a specific analytical method it had to be assured that all participants of the collaborative trial were familiar with the ELISA-method. At first the method description of the ELISA method already published (Hofmann, 1997) was finalised by taking into account results of the evaluation of the robustness of the method (e.g. impact of the extraction procedure and the pH-value on the result of the analysis). On the basis of this standard operation procedure (SOP) (von Holst, Honikel, Hofmann, Unglaub & Anklam, 1998) a video of the method has been produced explaining the most important steps of the method. Finally a workshop was held to discuss the execution of the method with the participants of the trial. The demonstration of the method was supported by showing the video.

Each participant received 8 samples (i.e. two samples of each material), labelled with a random number, thus ensuring that the identification of the samples were unknown to the investigating laboratories. Furthermore the laboratories were provided with one of the commercially available ELISA test kit, the SOP and a questionnaire to be filled in comprising all details of the conditions of the method and the results of the analysis.

2.2.2. Procedure

The whole procedure was carried out according to the method description of the producer of the ELISA kit for cooked pork ("Cooked Meat Species Identification Kit" Cortecs Diagnostics Ltd, Flintshire, UK) and the SOP (von Holst et al., 1998) established by the co-ordinators of the study. The method comprises the following steps.

2.2.3. Extraction of the samples

About 6 g of animal meal is mixed with about 20 ml of distilled water in a conical flask and allowed to swell for 15 min. After addition of 100 ml of physiological NaCl solution the mixture is held in the sealed flask in a boiling water bath for 15 min and shaken occasionally. The mixture is allowed to adjust to room temperature and then passed through a fluted filter. A slight turbidity of the solution, that can be observed occasionally, does not influence the results. Do not filter the solution twice. Finally the pH-value is measured. If necessary, the pH is adjusted to 7 ± 0.5 by taking an aliquot of 2 ml and adding 2 N HCl or 2 N NaOH to this aliquot (about 10 µl).

2.2.4. Enzyme immunoassay procedure

Subsequently an aliqout of the extract of the samples, the positive and the negative control are subjected to the immunoassay analysis according to the SOP. Finally the values of the optical density (OD) are measured at 405 or 415 nm using a microplate reader.

The following parameters are calculated using the OD of the positive control (PC), the negative control (NC) and the samples:

$$R\text{-value} = \frac{\mathrm{OD}_{\mathrm{sample}}}{\mathrm{OD}_{\mathrm{NC}}} \tag{1}$$

$$PK\text{-value} = \frac{(\mathrm{OD}_{\mathrm{sample}} - \mathrm{OD}_{\mathrm{NC}})}{(\mathrm{OD}_{\mathrm{PC}} - \mathrm{OD}_{\mathrm{NC}})} \cdot 100 \tag{2}$$

Table 2
Statistical evaluation of the homogeneity study

	R-value[a] General mean	*F*-value $_{\text{received}}$[b] ($F_{\text{critical}} = 3.02$)[c]	RSD (%) $_{\text{analytical}}$[d]
Animal meal I	1.1	0.67	3.2
Animal meal II	1.7	1.34	3.3
Animal meal III	2.1	1.12	2.1
Meat and bone meal IV (material prior to the subsequent sterilisation)	13	1.80	2.4

[a] *R*-value general mean: mean of all results ($n = 20$).
[b] *F*-value received: *F*-statistic received by comparing the within and between-bottle variance.
[c] F_{critical}: critical *F*-statistic indicating a significant difference of the variance; $F_{\text{critical, } \alpha = 0.05, \text{ degree of freedom} = 9,10} = 3.02$.
[d] RSD (%) $_{\text{analytical}}$: relative standard deviation representing the analytical error.

3. Results and discussion

3.1. General

All 21 participants completed the study in time. The results regarding the *R* and *PK* values are shown in Table 3. The participants were asked to use a bovine and chicken sample as negative control. Although the difference of the optical density regarding the bovine and chicken negative control turned out to be very low, the further evaluation of the data was performed using *R* and *PK*-values related only to the chicken negative control. This was done in order to exclude adverse effects of a different calculation basis on the *R*-value or *PK*-value respectively due to the use of different negative controls.

The estimates of the precision such as the average mean, and the standard deviation are obtained by a components of variance approach as proposed by the AOAC guideline (Association of Analytical Chemists, 1989). Robust statistics (Analytical Methods Committee, 1989; Lischer, 1987) have been applied to the estimation of the standard deviation and the average. The use of robust statistic shows some striking advantages (Thompson & Lowthian, 1996) in comparison to the traditional approach. The detection and rejection of outliers is not required, thus, the impact of extreme values on the average and the standard deviation is downweighted. Moreover robust statistic works well for distributions that deviate from normal distribution due to extreme values which is typical for data received in a collaborative trial. In this study the algorithm recommended by the Swiss Food Manual (SLB, 1989) is used which is implemented via an Excel® macro.

3.2. Examination of the R-values

The statistical analysis of the trial is shown in Table 4. The first column contains the target mean of the *R*-value of the test materials as determined by three independent expert laboratories not participating in the collaborative trial. This value is used to check for a significant bias between the robust mean of the results of all participants shown in the second column and the target value. By taking into account the confidence range of the mean deduced from the *t*-distribution no significant difference between the target mean and the robust mean of the trial can be observed indicating that the method fulfils the criterion of an absence of bias.

In the third and fourth column the within-laboratory standard deviation (s_r) and the within-laboratory relative standard deviation (RSD_r) of the *R*-value are shown. These values represent the variability that can be expected if a sample is analysed by the same laboratory in replicates. The RSDr ranged from 3 to 6% depending on the *R*-value.

The s_R and the corresponding RSD_R (or CV) reflect the variability of analytical results of the method between laboratories. The RSD_R ranged from 11 to 22%. Samples with a low *R*-value are attributed with a lower standard deviation which fits to the specific purpose of the method as the sufficient heat treatment of

Table 3
Collaborative results of the ELISA method (*R* and *PK*-values)

Laboratory no.	Animal meal I		Animal meal II		Animal meal III		Meat and bone meal (unsterilised)	
	R-value	*PK*-value	*R*-value	*PK*-value	*R*-value	*PK*-value	*R*-value	*PK*-value
1	1.13/1.23	1.19/1.67	2.15/1.87	6.7/8.8	2.41/1.69	10.3/5.0	12.8/13.3	90/87
2	1.07/1.04	0.47/0.28	1.85/1.96	6.2/5.5	2.07/2.08	6.9/7.0	14.6/14.6	88/88
3	1.09/1.11	1.16/0.94	1.88/1.72	7.3/5.6	1.96/2.08	11/9.8	10.2/10.3	94/95
4	0.99/1.04	0.35/0.00	1.71/1.55	7.5/6.1	2.23/2.20	9.9/10.3	12.8/13.0	100/102
5	1.10/1.11	0.81/0.89	1.68/1.66	5.4/5.3	2.1/1.88	7.0/8.8	12.4/12.7	91/93
6	1.09/1.15	0.70/1.16	1.82/1.77	6.2/5.8	2.17/2.16	8.9/8.8	13.0/13.4	90/94
7	1.03/1.07	0.27/0.59	1.69/1.87	6.1/7.7	2.02/2.09	9.0/9.7	12.0/12.3	98/100
8	1.20/1.26	1.06/1.42	2.92/2.13	9.3/6.2	3.20/3.11	13/12	15.0/17.9	77/91
9	1.16/1.24	1.13/1.69	1.88/1.81	5.6/6.1	2.33/2.33	9.2/9.2	14.7/15	95/97
10	1.11/1.09	0.67/0.59	1.99/2.03	6.0/6.3	3.30/2.54	14/9.4	16.0/15.0	91/86
11	1.07/1.05	0.47/0.53	1.71/1.71	5.8/5.8	2.20/2.16	9.9/9.5	12.7/13.2	96/101
12	1.10/1.10	0.40/0.64	2.10/2.20	5.4/5.9	2.80/2.70	8.6/8.5	22.0/23.0	106/108
13	1.10/1.09	0.45/0.50	1.72/1.94	5.1/4.7	2.4/2.74	9.4/7.5	15.8/16.0	79/80
14	1.06/1.08	0.58/0.70	1.56/1.62	5.7/5.1	1.92/1.94	8.7/8.5	11.0/11.0	92/93
15	1.32/1.20	2.19/1.36	2.08/2.08	7.4/7.4	2.36/2.24	8.2/8.6	13.0/13.2	83/84
16	1.00/0.90	−0.4/−0.5	1.40/1.50	3.7/4.4	1.60/1.90	5.4/7.6	8.2/8.6	62/66
17	0.93/0.91	−0.88/−0.88	1.36/1.36	3.4/3.2	1.69/1.67	6.3/6.1	9.4/9.4	78/78
18	1.17/1.17	1.1/1.1	1.90/1.93	5.5/5.3	2.72/2.72	10/10	15./15.7	85/87
19	1.17/1.50	0.60/2.88	2.11/1.92	6.1/5.2	2.47/2,49	8.1/8.3	18.0/16	98/84
20	0.97/1.15	0.33/1.50	1.60/1.50	5.5/4.6	1.80/1.70	7.8/6.9	10.3/10.6	91/93
21	1.21/1.19	1.16/1.10	1.99/2.10	6.3/5.6	2.68/2.40	9.6/7.9	17.5/16.7	93/89

Table 4
Statistical analysis of collaborative results for the *R*-value of the ELISA method

	Target mean	Mean of the trial	s_r	RSD_r (%)	s_R	RSD_R (%)
Animal meal I	1.05	1.1 ± 0.05	0.05	4	0.12	11
Animal meal II	1.77	1.83 ± 011	0.1	5.5	0.25	14
Animal meal III	2.1	2.2 ± 0.22	0.13	6	0.45	20
Meat and bone meal (material prior to the subsequent sterilisation)	14	13 ± 1.3	0.4	3	2.9	22

[a] s_r = within-laboratory standard deviation representing the analytical error.
[b] RSD_r = within-laboratory relative standard deviation.
[c] s_R = between-laboratory standard deviation.
[d] RSD_R = between-laboratory relative standard deviation.

animal meal is proved when the *R*-value is low. The between laboratory standard deviation reflects the variability of the method and has to be taken into account for the establishment of the threshold limit of the test which is used to differentiate between sufficient and non-sufficient heat treatment of the material. As shown in the Table 4 *R*-values of the animal meal samples I and II are 1.1 and 1.8 respectively. As the unambiguous differentiation of animal meals treated under different conditions is crucial for the purpose of the method it was examined whether the observed difference of these *R*-values is significant by taking into account the RSD_R of the method. The *t*-test was employed showing that the difference of the average values related to animal meal I and II is highly significant (99% confidence interval).

The distinct differentiation of these materials is confirmed by a visual examination of the mean value of the results of all laboratories as depicted in Fig. 1.

3.3. *Examination of the* PK-*values*

The corresponding results of the evaluation of the *PK*-values are shown in Table 5. The relative standard deviation between laboratories related to animal meal **I** is very high (about 83%) and *decreases* to 9% if the corresponding *PK* value is very *high*. From this dependency it can be concluded that low *PK*-values are characterised by a high variability of the data. A comparison of the evaluation regarding the *R*-value shown in Table 4 revealed that the opposite relationship applies to the *R*-value: the standard deviation of the data related to low *R*-values is about 11% and increases to 22% when the *R*-value reaches the corresponding value of the positive control. In order to verify whether this result is purely coincidental or reflects a characteristic of these measures, the law of error propagation was applied to the equations for the calculation of the *R*-value and *PK*-value [Eqs. (1) and (2)]. The elaboration confirmed the results achieved in this study: If the optical density (OD) of the samples is assumed to be low (i.e. in the magnitude of the negative control) then the *R*-value shows a lower coefficient of variation than the *PK*-value whilst the opposite holds true if the OD is in the range of the positive control.

However, the higher relative standard deviation of the *PK*-values which is shown to be an intrinsic property of equation 2 does not diminish the applicability of this parameter to detect non-proper heat treatment. This is demonstrated by comparing the results of the laboratories regarding the *PK*-value of AM I and AM II as shown in Fig. 2. Obviously the large difference of the

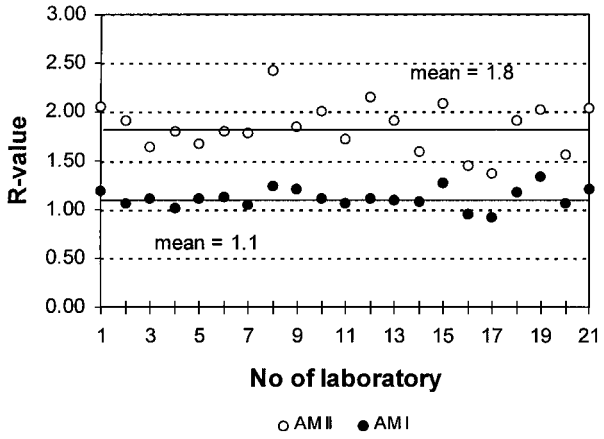

Fig. 1. *R*-values of the participating laboratories regarding the test material AM I and AM II.

Table 5
Statistical analysis of collaborative results for the *PK*-value of the ELISA method

	Mean of the trial	s_r[a]	RSD_r (%)	s_R	RSD_R (%)
Animal meal I	0.80	0.26	35	0.7	83
Animal meal II	5.8	0.6	12	1.4	24
Animal meal III	8.7	0.7	8	1.7	20
Meat and bone meal (material prior to the subsequent sterilisation)	90	2.3	3	8	9

[a] For abbreviations, see Table 4.

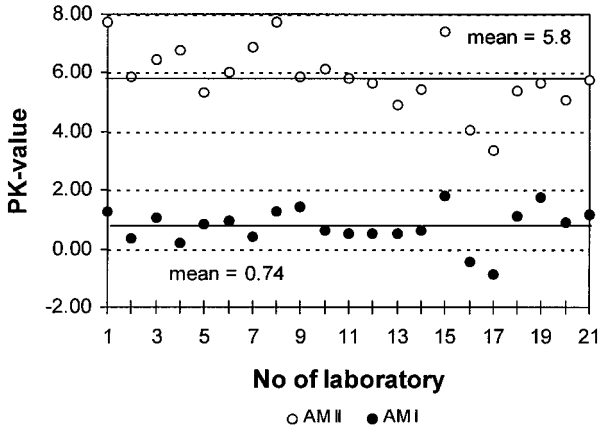

Fig. 2. *PK*-values of the participating laboratories regarding the test material AM I and AM II.

PK-values of AM I and AM II respectively compensates for the high standard deviation reflecting the variability of the data between the laboratory. The distinct differentiation is also supported by the t-test indicating that the difference regarding the *PK*-value of AM I and AM II is significant on the 99% confidence level.

The examination of the results of the laboratories confirms that the *R*-value as well as the *PK*-value are suitable parameters to investigate the appropriate heat treatment of animal meal.

3.4. Distribution of the data

The coefficient of variation (CV) regarding the *R*-value and *PK*-value estimated in this study is required to set a sound threshold for the proper heat treatment since the CV reflects the unavoidable variability of the method. In general it is assumed that this variability is caused by random errors resulting in a normal distribution of the data. Since the standard deviation of a method is a measure for the uncertainty of the result of the analysis of an animal meal sample, the RSD_R of the respective parameter has to be taken into account in order to establish a threshold limit of this parameter. This threshold value should allow an unambiguous identification of animal meal that has not been treated according to the regulation. In general the RSD_R is used to estimate the 95% confidence interval of the *R*-value and *PK*-value respectively. Since the underlying assumption of this approach is the normal distribution of the data, a check for non-normality of these parameters of all materials was carried using the Kolmogorov-Smirnov test (Thompson & Lowthian, 1996). This test was carried out after having discarded extreme values outside the range of $\pm 2 \cdot RSD_R$. The evaluation of the results of this test indicates, that the *R*-value and *PK*-values of all materials pass the test for normal distribution.

4. Conclusions

The ELISA method is a reliable method for the surveillance of the appropriate heat treatment of animal meals, as demonstrated by the results of a collaborative trial. The statistical analysis of the results of the participants confirm that the sensitivity of the method is sufficient to detect a divergent treatment of animal waste from the target sterilisation conditions. Due to the fact that the results of the laboratories are normally distributed, a 95% confidence interval regarding the *R*-value and *PK*-value can be calculated.

In the second part of the study trials will be performed in order to set a limit of the *R*-value and *PK*-value indicating the proper heat treatment of the animal meal. These trials include the investigation of animal meal produced in a rendering plant using a temperature probe capable of remote measurements of the temperature in the autoclave. Moreover other methods of the processing of animal waste such as the continuous techniques will be examined as well. Finally the values for the *R*-value received in these experiments and the 95% confidence level of the method will be used to establish the tolerance level for the *R*-value and *PK*-value.

5. Collaborators in the study

G. Spadinger, Bundesamt und Forschungszentrum fuer Landwirtschaft, Austria; J. Rentsch, Federation of Migros Cooperatives, Switzerland; S. Pecoraro, Bayerische Landesanstalt fuer Ernaehrung, Germany; M. Krause, Plantedirektoratet, Denmark; L Pallaroni and M. Moschini Univers. Piacenza, Italy; M. Ansfield, Veterinary Investigation Centre, UK; B. Poepping, MAFF-CSL, UK; W. Gaede, LV-LUA Stendal, Germany; S. Marmo, Plant Production Inspection Center, Finland; D. Hayes, State Laboratory, Eire; T. Jemmi, Bundesamt fuer Veterinaerwesen, Switzerland; J.M. Nunes da Costa, LNIV, Portugal; F. Lee, LGC, UK; S. Pryde, Rowett Research Institute, UK; C. Cordes, Wiertz/Eggert/Jörissen GmbH, Germany; J. Bosch Laboratori Agroalimentari De La Generalitat De Catalunya, Spain; R. Ziebal Laboratoire DGCCRF, France; S. Pfeifer, Tiergesundheitsdienst Bayern e.V., Germany; K. Cerenius, Analysen Nordic AB, Sweden; M. Tapia Ministerio De Agricultura, Pesca Y Alimentacion, Spain.

Acknowledgements

The authors wish to thank L. Meyer and P. Engstler for the technical support regarding the homogeneity study. Furthermore the authors would like to thank the heads of the meat and bone meal plant "Ed. Geistlich Soehne AG", Switzerland and of the rendering plants

''Zweckverband Tierkoerperbeseitigung Warthausen'', Germany, Zweckverband Protec Orsingen'', Germany for the provision of the test material which was kindly supported by E. Gantert (Ltd. MR, Oberste Landesbehoerde Baden-Wuerttemberg, Germany).

Y. Diehl and D. Thiele (Transia GmbH, Germany) are acknowledged for their helpful discussions regarding the refinement of the method description.

Finally we are grateful to DG XXIV, European Commission (EC) for financial support reflecting the pivotal role of consumer protection in the policy of the EC. Several measures have been taken by the EC to minimise the risk to the consumers associated with the emergence of mad cow disease. Since the origin of BSE is supposed to be strongly connected to the feeding of cattle with compound feed containing animal meal of mammals the EC has released decisions regarding the composition and quality of feedingstuff in order to avoid a further transmission of the causative pathogen of the BSE disease. Moreover the additional funding of this study by DG XXIV puts an emphasise on the fact that analytical methods validated at international level are urgently required to enforce European law.

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