EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Institute for Reference Materials and Measurements

RUGGEDNESS STUDY OF IMMUNOASSAYS FOR PROCESSED ANIMAL PROTEINS DETECTION IN FEED:

Neogen Reveal for Ruminant Feed Test System

A. Boix, F. Serano, S. Bellorini, C. von Holst
European Commission. DG JRC-IRMM, Geel, Belgium

GE/R/FSQ/03/2006/09/04

The mission of the IRMM is to promote a common and reliable European measurement system in support of EU policies.

European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
Ana Boix
European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg 111
B-2440 Geel • Belgium

Email: ana.boix-sanfeliu@ec.europa.eu
Tel.: +32 (0)14 571 227
Fax: +32 (0)14 571 787

http://www.irmm.jrc.be
http://www.jrc.ec.europa.eu

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Institute for Reference Materials and Measurements

RUGGEDNESS STUDY OF IMMUNOASSAYS FOR PROCESSED ANIMAL PROTEINS DETECTION IN FEED:

Neogen Reveal for Ruminant Feed Test System

A. Boix, F. Serano, S. Bellorini, C. von Holst

European Commission. DG-JRC-IRMM, Geel, Belgium

GE/R/FSQ/03/2006/09/04

Table of contents

1. Introduction 1
2. Principle of the method 2
3. Sample preparation and analysis 2
4. Analysis of results 4
5. Conclusions 9
6. Annexes 10

1. Introduction

The development and validation of ruminant specific tests has highest priority, especially to solve problems regarding traces of meat and bone meal (MBM) in feed at extremely low levels. In 2004 the Institute for Reference Materials and Measurements (IRMM) of the European Commission's Joint Research Centre conducted on behalf of DG Health and Consumer Protection (DG-SANCO) a pre-validation study of immunoassays[1]. The major objective of the study from 2004 was to assess whether the currently commercially available immunoassays tests could play a major role in future control activities of Member States' official laboratories, allowing for an animal species specific test for the detection of MBM in animal feed. The study revealed the potential of two existing methods (one dip stick method developed by Neogen and one ELISA developed by Antibodyshop) for the intended purpose which was the detection of processed animal proteins (PAPs) from ruminants in feed.

As a follow-up of the study from 2004 the IRMM conducted a ruggedness study of these immunoassays in order to establish the impact of various feed ingredients on the analytical results and to evaluate the transferability of the methods from the laboratory that developed the test to another laboratory before conducting a full validation of the methods through an interlaboratory study. This report presents the results of the ruggedness test of the Neogen Reveal for Ruminant Feed Test System The ruggedness test was performed by applying the method on quite different and real world feed samples, either without PAPs or fortified with PAPs from various animals. In particular it was of interest to know, which of the samples containing ruminant PAPs were correctly classified as positive or wrongly classified as negative. In addition, we also measured the feed samples containing PAPs from animals *other* than ruminants to evaluate possible cross-reactivity due to the presence of these PAPs in the test samples.

In the case the method passed the ruggedness test described in this study, the validation of the method by conducting an intercomparison study at European level could be considered.

---

[1] *Boix et al. (2004). Determination of processed animal proteins (PAPs) including meat and bone meal (MBM) in feed. Administrative Arrangement No.SANCO/17 04 02/04/SI2.373351, Final report, 102 pp. http://ec.europa.eu/comm/food/food/biosafety/bse/bse51_en.pdf.*

2. Principle of the method

Reveal for Ruminant in Feed is a single-step lateral flow immunochromatographic assay. The extract is wicked through a reagent zone, which contains antibodies specific for heat stable ruminant muscle protein, conjugated to colored particles. If ruminant by-product is present, it will be capturated by the conjugated antibodies. The ruminant-antibody-particle complex is then wicked onto a membrane which contains a zone of antibody specific for the ruminant muscle protein. This zone captures the complex allowing the particles to concentrate and form a visible lint. If no ruminant by-product is present, no line will form. The membrane also contains a control zone where an immune complex present in the reagent zone is captured by an antibody, forming a visible line. The control line will always form regardless of the presence of ruminant by-product, ensuring the strip is working properly. The test strip provides clear results in approximately 15 minutes The analytical result of the dip stick can be visually evaluated or by using a dip stick reader. In this study the "Accuscan" instrument from Neogen was utilised to measure the response of the dip stick.

3. Sample preparation and analysis

The test materials utilised in the study, were a set of representative samples of typical feed ingredients and compound feeds for farm animals (Table 1) and were provided by the European Feed Manufacturers Federation (FEFAC) and were obtained from a feed mill with high quality standard which performs its own check by classical microscopy to ensure that no MBM is present. The compound feeds consisted in seventeen different compound feeds for bovine and fifteen different compound feeds for porcine and chicken containing typical feed ingredients. The composition of the compound feeds used in the study is shown in Annex I, II and III.

All blank samples (47 different compound feeds and 13 feed ingredients) were analysed to assess the rate of false positive results of the method. In addition, the samples were fortified with PAPs from various animals at different concentration levels in order to assess the sensitivity of the method and the cross reactivity of the immunoassay towards PAPs from animals other than ruminants. The following types of samples were prepared: i) all feed ingredients were fortified with pure bovine MBM at 0.1% and 0.5% level, ii) all compound feeds were fortified with porcine MBM at 5% level and iii) four compound feeds intended for pig, chicken and cattle were fortified with fishmeal at 5% level.

The pure bovine and porcine meat and bone meals were obtained from a pilot plant and produced from species pure by-products of each considered animal species (cattle, pig). The meals were treated at 133 °C and 3 bar for 20 minutes. Afterwards the material was dried under atmospheric conditions until the moisture content was below 10%. Finally the product was pressed and ground. The samples were analysed by Polymerase Chain Reaction (PCR) in order to establish the species included in the sample. The results indicated that the bovine and porcine materials contain respectively only bovine and porcine materials.

The fishmeal was obtained directly from a fishmeal producer. The fishmeal was analysed by PCR in order to check the species composition in the sample. The results indicated that the fishmeal did not contain bovine, porcine and poultry materials.

Table 1. Feed ingredients and compound feeds included in the study

Composition	*Sample Code*
Compound feeds	
Pigfeeds	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15
Chickenfeeds	18,19,20,21,22,23,24,25,26,27,28,29,30,31,32
Cattlefeeds	68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84
Feed ingredients	
Manioc	16
Maize	34
Soybeanmeal	37
Barley	40
Wheatglutenfeed	43
Cornglutenfeed	46
Rapeseedmeal	50
Citruspulp	53
Beetpulp	56
Molasses	59
Palmoilfat	62
Skimmed milkpowder	65
Cotton	90

Since the MBM was introduced into the test material by fortification of individual samples with very small amounts of the MBM (e.g. 10 mg of MBM in 10 g of compound feed), it was important to assure sufficient homogeneity of this material. Therefore the pure bovine MBM was divided in four granulometric fractions (<100µm, 100-200µm, 200-400µm and >400µm). Three sub samples (100mg) of each fraction were extracted and tested according to the protocol provided by Neogen (see Annex IV). An AccusScan Reader (a lateral flow test reader combined with a personal data assistant and data management software) was used to obtain an quantitative estimate of the immune response and to objectively analyse the results. A single factor ANOVA was applied to check the homogeneity of the material. The results confirmed that the immunoassay response was very similar amongst the various sieve fractions thereby confirming sufficient homogeneity of the pure cattle MBM.

In addition, a set of test materials (Table 2) already used in the pre-validation study of immunoassays performed by IRMM in 2004[1] was provided by the Walloon Agricultural Research Centre (CRA-W). The base of these test materials was a compound feed for bovine containing typical feed ingredients. The compound feed was tested by PCR, immunoassay kits and Near-Infrared Microscopy (NIRM) in order to check absence of contamination with PAPs. The results of the analyses were negative for all methods thereby confirming its blank status with respect to the presence of PAPs.

Table 2. Samples included in the pre-validation study of immunoassays performed in 2004 and utilised also in the present study: compound feed for bovine with different MBMs at various concentrations.

	DQ-04-0267-01	DQ-04-0267-09	DQ-04-0267-10	DQ-04-0267-12
	Concentration (%)			
MMBM (bovine + porcine)	-	0.1	-	-
Bovine MBM 134°C	-	-	0.1	-
Porcine MBM 133°C	-	-	-	0.5
Fishmeal	-	5.0	-	-

The mammalian meat and bone meal (MMBM) used in the preparation of the fortified test material as shown in Table 2 was a mix of bovine and porcine processed material. This MMBM was analysed by PCR in order to check the species present in the sample, confirming that the samples mainly contained bovine porcine materials with low amounts of poultry meal. The content of bovine MBM was about 45 %. The bovine and porcine MBMs and the fishmeal were the same used in this study for the fortification of the feed ingredients and compound feeds.

All samples fortified or not with PAPs from different species were extracted and analysed according to the procedure described in the protocol provided by Neogen (see Annex IV). An AccusScan Reader provided by Neogen was used to objectively interpret the results. By fully inserting a lateral flow test into the reader, the instrument takes a digital photograph of the test strip's result area. The image is then analysed pixel by pixel. If a test line is detected, a positive or negative results is returned, along with a line intensity score of 0, 0.5, 1, 2, 3 or 4. The value "0" is read as negative, wherease the values from 0.5 to 4 indicate the presence of ruminant PAPs.

4. Analysis of results

<u>*Analysis of feed ingredients*</u>

Blank feed ingredients were analysed in duplicates. Only in the cases where a false positive result was obtained, the samples were analysed again in triplicates. The results are shown in Table 3.

All test materials except two feed ingredients (citruspulp and beetpulp) which gave a false positive result when analysed as a blanks, were fortified with bovine meat and bone meal at 0.1 % level (target concentration of MBM in feed). Only four samples out of eleven were correctly classified as positives. It is important to state that this level of contamination with MBM is below the limit of detection (LOD) stated by the manufactures which is 1 % ruminant by-products in feed. Feed ingredients containing MBM at 0.1 % which were wrongly classified as negative were fortified with bovine MBM at 0.5 %. This time, all materials gave a positive response, proving that the LOD is between 0.1% and 0.5% ruminant by-products in feed. Additionally, the samples which gave a false positive response (citruspulp and beetpulp), were analysed by classical microscopy (according Directive (EC) No 2003/126) and near-infrared microscopy (NIRM) with a negative result in both cases. Nevertheless, false positive results in tuber and root crops (i.e. beet pulp) could be explained by the presence of traces of MBM present in the soil due to fertilization[2].

Table 3. Results of the analysis of blank feed ingredients and the same materials fortified with bovine MBM at two concentration levels. Samples indicated as (n.a.) were not fortified with MBM and therefore not analysed, since the blank samples already gave a positive response.(+) sample classified as positive with the indication of the figure given by the Accuscan Reader. (-) sample classified as negative.

Feed Ingredients	**Blank Samples**						**Spiked Samples**					
(sample codes)							**0.1% Bovine MBM**			**0.5% Bovine MBM**		
	Replicates					Final Result	Replicates		Final Result	Replicates		Final Result
	1	2	3	4	5		1	2		1	2	
Manioc (16)	-	-				-	+	-	-	+	+	+
Maize (34)	-	-				-	-	-	-	+	+	+
Soybeanmeal (37)	-	-				-	-	-	-	+	+	+
Barley (40)	-	-				-	-	-	-	+	+	+
Wheatglutenfeed (43)	-	-				-	+	+	+			
Cornglutenfeed (46)	-	-				-	+	+	+			
Rapeseedmeal (50)	-	-				-	+	+	+			
Citruspulp (53)	-	+ 2	+ 2	+ 1	+ 2	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Beetpulp (56)	+ 2	+ 1	+ 0.5	+ 3	+ 4	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Molasses (59)	-	-				-	-	-	-	+	+	+
Palmoilfat (62)	-	-				-	-	-	-	+	+	+
Skimmed milkpowder (65)	-	-				-	-	-	-	+	+	+
Cotton (90)	-	-				-	+	+	+			

<u>*Analysis of compound feeds*</u>

Compound feeds intended for cattle, pig and chicken, were analysed as a blank samples in duplicates. Only in the cases where a false positive result was obtained, more replicates were analysed. The results are shown in Tables 4 to 6.

All chickenfeeds (Table 4) analysed as blanks or fortified with porcine MBM or fishmeal at 5% level were correctly classified as negatives.

---

[2] EC Regulation 1292/2005 amending Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards animal nutrition. Official Journal of the European Communities. 06.08.2005. L205 3-11.

Table 4. Results of chickenfeed samples analysed as blanks and fortified with porcine MBM and fishmeal.

Chickenfeeds	**Blank Samples**			**Spiked Samples**					
(sample codes)				**5 % Porcine**			**5 % Fishmeal**		
	Replicates		Final Result	Replicates		Final Result	Replicate s		Final Result
	# 1	# 2		# 1	# 2		# 1	# 2	
18	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-			
22	-	-	-	-	-	-			
23	-	-	-	-	-	-			
24	-	-	-	-	-	-			
25	-	-	-	-	-	-			
26	-	-	-	-	-	-			
27	-	-	-	-	-	-			
28	-	-	-	-	-	-			
29	-	-	-	-	-	-			
30	-	-	-	-	-	-			
31	-	-	-	-	-	-			
32	-	-	-	-	-	-			

In the case of pigfeeds (Table 5), two samples out of fifteen gave a false positive response when analysed as a blanks (#2 & #15) or fortified with pig MBM at 5% level (#4 & #6). Three random selected samples were fortified with fishmeal at 5% level. In this case one sample out of three gave a false positive response. Additionally, the blank samples which gave a false positive response were analysed by classical microscopy (according Directive (EC) No 2003/126) and NIRM with a negative result in both cases. Typical ingredients of compound feed were evaluated against the possibility of introducing ruminant tissue in the feed that could lead to a false positive result. In a former study[3] it was shown that ruminant fat could be identified by the same immunoassay as validated in this study due to protein traces present in the residual insoluble impurities (RIIs) of the fat. The identification of tallow by immunoassay was even possible when the fat did not contain more than 0.15 % RIIs and when the tallow was mixed into lard. Therefore false positive results could be explained by the presence of animal fat and beetpulp in the composition of pigfeeds, since these ingredients were present in the formulation of all samples that gave a false positive response (see Annex III).

---

[3] *Bellorini S, Strathmann S, Baeten V, Fumiere O, Berben G, Tirendi S, von Holst C (2005) Discrimination of animal fats and their origins: assessing the potentials of Fourier transform infrared spectroscopy, gas chromatography, immunoassay and polymerase chain reaction techniques. Anal. Bioanal Chem 382, 1073-1083.*

Table 5. Results of the analysis of pigfeed samples and the same materials fortified with porcine MBM or fishmeal. Samples indicated as (n.a.) were not fortified with MBM or fishmeal and therefore not analysed, since the blank samples already gave a positive response.(+) sample classified as positive with the indication of the figure given by the Accuscan Reader. (-) sample classified as negative.

Pigfeeds	**Blank Samples**							**Spiked Samples**					
(sample codes)								**5 % Porcine MBM**			**5 % Fishmeal**		
	Replicates						Final Result	Replicates		Final Result	Replicates		Final Result
	# 1	# 2	# 3	# 4	# 5	# 6		# 1	# 2		# 1	# 2	
1	-	-					-	-	-	-	-	-	-
2	+ 1	+0.5	+0.5	+ 1			+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	-	-					-	-	-	-	-	-	-
4	-	-					-	-	+0.5	+	+0.5	+1	+
5	-	-					-	-	-	-			
6	-	-					-	+0.5	+1	+			
7	-	-					-	-	-	-			
8	-	-					-	-	-	-			
9	-	-					-	-	-	-			
10	-	-					-	-	-	-			
11	-	-					-	-	-	-			
12	-	-					-	-	-	-			
13	-	-					-	-	-	-			
14	-	-					-	-	-	-			
15	-	+ 1	-	+ 1	-	+ 1	+	n.a.	n.a.	n.a.			

Six cattlefeeds out of seventeen were wrongly classified as positives when compound feeds for cattle were analysed as a blank. These false positive results could be explained by the presence of citruspulp and beetpulp in the composition of the cattlefeeds which have been tested positive (Table 3). Additionally, the samples which gave a false positive response when analysed as a blank were analysed by classical microscopy (according Directive EC2003/126) and NIRM with a negative result in both cases except for sample number 80 in which two animal particles were detected by classical microscopy.

Cattlefeeds correctly classified as negatives when analysed as a blank were fortified with porcine MBM at 5% level (see Table 6). Only four out of twelve samples were correctly classified as negatives. All samples fortified with fishmeal gave false positive results (3 out of 3).

The samples previously used in the prevalidation study of immunoassays carried out in 2004 were all correctly classified (see Table 7), except the material containing MMBM. This result, however, does not question the performance of the test since this material only contains about 0.05% bovine material, which is below the target level of 0.1% PAPs in the material.

Table 6. Results of the analysis of cattlefeed samples and the same materials fortified with porcine MBM or fishmeal. Samples indicated as (n.a.) were not fortified with MBM or fishmeal and therefore not analysed, since the blank samples already gave a positive response.(+) sample classified as positive with the indication of the figure given by the Accuscan Reader. (-) sample classified as negative.

Cattle feeds	**Blank Samples**								**Spiked Samples**					
(sample codes)									**5 % Porcine MBM**			**5 % Fishmeal**		
	Replicates							Final Result	Replicates		Final Result	Replicates		Final Result
	# 1	# 2	# 3	# 4	# 5	# 6	# 7		# 1	# 2		# 1	# 2	
68	-	-			-			-	+2	+1	+	+1	+1	+
69	-	-			-			-	+1	+1	+	+1	+1	+
70	-	+0.5	-	+1	-			+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
71	-	-			-			-	+1	+3	+	+1	+1	+
72	-	-						-	-	-	-			
73	-	-						-	+1	+1	+			
74	-	-			-			-	+1	+1	+			
75	-	-			+1	+1	+1	+	+1	+1	+			
76	-	-						-	-	-	-			
77	-	+1	-	+2	-	+0.5		+	n.a.	n.a.	n.a.			
78	-	-						-	+1	+0.5	+			
79	-	-			-			-	+1	+1	+			
80	-	+1	+1	+1	+1			+	n.a.	n.a.	n.a.			
81	-	+1	-	+2	+2			+	n.a.	n.a.	n.a.			
82	-	+ 1	-	+ 1				+	n.a.	n.a.	n.a.			
83	-	-						-	-	-	-			
84	-	-						-	-	-	-			

Table 7. Results of samples from the prevalidation study on immunoassays carried out in 2004 (Boix et al, 2004). (+) sample classified as positive with the indication of the figure given by the Accuscan Reader.

Samples from Prevalidation Study Immnunoassays			
Composition	Replicates		Final Result
	# 1	# 2	
0 % MBM	-	-	-
0.1% MMBM + 5% fishmeal	-	-	-
0.1% bovine MBM 134 °C	+1	+1	+
0.5 % porcine MBM 133°C	-	-	-

When pooling the results from all materials containing ruminant MBM at 0.1 %, 7 out of 13 amples were wrongly classified as negative. However, all false negative samples were tested correctly positive when they contained 0.5 % of MBM.

5. Conclusions

A set of representatives' samples of typical feed ingredients and compound feeds for farm animals has been analysed by using the commercially available test kit developed by Neogen "*Reveal for Ruminant Feed Test System*".

From the results we draw the following conclusions:

- This test is a valuable tool for the detection of ruminant by-products in feed because of their easy handling and short analysis time. The sensitivity of the test is between 0.1% and 0.5% ruminant by-products in feed.
- False positive results in feeds were related to the presence of beetpulp or citruspulp or animal proteins in the composition of compound feeds.
- The presence of some feed ingredients such as beetpulp or citruspulp in the formulation of compound feeds might be a source of false positive results in feeds.
- The presence of animal fats from the rendering industry might be a source of false positive results in some compound feeds, especially in pig feeds where this animal fat is usually present.
- The test shows sufficient sensitivity at the level of 0.5 % of ruminant MBM but insufficient sensitivity when the samples contained 0.1 % of ruminant MBM.
- Some of the blank animal feed samples were wrongly specified as positive. However, this aspect does not pose a major problem when integrating the method in a global control system, applying the dipstick mainly for screening purposes. Positive samples would then need to be tested by a confirmatory method.

Acknowledgements

The authors would like to thank A. Swinkels, (NUTRECO, The Netherlands), S. Woodgate (EFPRA, Belgium), A. Döring (FEFAC, Belgium) and V. Baeten (CRA-W, Belgium) for providing us with the feed samples used in the study. We are grateful to H. Emons, F. Ulbert and H. Emteborg (RM Unit of IRMM) for helpful discussion regarding the homogeneity testing of the MBM used in this study and for conducting the sieve analysis. The authors are also grateful to F. Klein and R.Langley (Neogen) for providing the test kits and the reader used in the ruggedness test and for the constructive discussions regarding the extraction procedure.

6. Annexes

***Annex I. Composition of cattlefeeds used in the study*.**

Sample codes	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**
Toasted Soyameal (GMO)	√	-	-	√	√	-	-	√	√	√	√	√	√	-	-	-	-
Palmkernel meal	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	-	-
Cabbage and turnip seeds by products	√	√	√	√	√	√	√	√	√	√	-	√	√	-	√	-	-
Toasted Soyameal	-	√	-	-	√	-	-	-	-	-	√	√	-	-	-	-	-
No bitter lupin	-	-	√	√	√	-	-	-	√	-	√	√	-	-	-	-	-
Calcium carbonate	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	-	-
Beet vinasses	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	-	-
Cane molasses	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	-	-
Salt	-	-	-	-	√	-	-	√	-	-	√	√	-	√	√	-	-
Magnesium oxide	√	√	√	√	√	√	-	√	-	√	√	√	√	√	√	-	-
Citrus pulp	√	√	√	√	-	√	√	√	-	√	√	√	√	√	√	-	-
Maize (GMO)	√	√	√	√	-	√	-	-	-	√	√	√	√	-	√	-	-
Rye	-	-	-	-	-	√	-	-	-	√	-	-	-	√	-	-	-
Beet pulp	-	√	√	√	-	√	√	√	√	-	√	√	√	√	-	√	-
Soyabean hulls (GMO)	√	√	√	√	-	√	√	-	√	√	-	√	√	√	√	-	-
Potato fruit-juice, concentrated	√	-	√	√	-	√	√	√	√	√	-	√	√	√	-	-	-
Liquid lactose	-	-	√	-	-	√	√	√	-	√	-	√	-	√	-	-	-
Vegetable fatty acids	√	√	√	√	-	√	√	√	√	√	√	√	√	√	√	-	-
Wheat semolina	√	√	√	√	-	√	√	√	√	√	√	√	√	√	√	√	-
Triticale	√	-	-	√	-	-	√	-	√	-	√	-	√	-	√	-	-
Beet molasses	-	-	-	-	-	-	√	-	-	√	-	√	-	√	-	√	-
Coconut, extracted	√	-	√	√	-	-	√	√	-	√	-	√	√	-	√	-	-
Maize	-	√	-	-	-	-	-	-	-	-	√	-	-	-	√	√	-
Wheat	-	-	√	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Soya flour	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√	√
Wheat gluten feed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Garden peas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sunflower seed, extracted	-	-	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-
Carobs	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√	-
Animal fat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cottonseed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√	√
Monocalciumphosphate	-	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	-
Manioc	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maize grits	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√	-
Wheat bran	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√
Sunflower flour	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√
Maize germ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√
Fish meal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maize gluten feed	√	-	-	-	-	-	-	√	√	-	-	-	-	-	-	-	-
Propylenglycol	-	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	-
Potato protein	-	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	-
Maize gluten feed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√	-

Annex II. Composition of pigfeeds used in the study.

Sample codes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Toasted Soyameal	√	-	-	-	√	√	√	√	-	√	-	-	-	-	√
Palmkernel meal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cabbage and turnip seeds by products	√	√	√	√	√	√	√	√	√	√	-	√	√	√	√
Toasted Soyameal	-	-	√	√	√	-	-	-	√	-	√	-	√	-	-
No bitter lupin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Calcium carbonate	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Beet vinasses	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cane molasses	√	√	√	√	√	√	√	√	-	√	-	√	√	-	√
Salt	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Magnesium oxide	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrus pulp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maize	√	-	-	√	√	-	√	√	√	√	-	-	√	√	-
Rye	√	√	-	√	-	√	√	-	√	√	-	√	√	√	√
Beet pulp	-	√	√	√	-	√	-	-	√	-	-	√	√	√	-
Soyabean hulls	-	√	√	√	√	√	-	√	-	-	-	√	-	-	√
Potato fruit-juice, concentrated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Liquid lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vegetable fatty acids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wheat semolina	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Triticale	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beet molasses	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coconut, extracted	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maize	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wheat	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Barley	√	√	√	√	√	-	√	√	√	√	-	√	√	√	√
Wheat gluten feed	√	-	√	-	√	-	√	-	√	√	-	√	√	√	√
Garden peas	√	-	√	√	√	√	√	√	√	√	-	√	√	√	√
Sunflower seed, extracted	√	√	√	√	√	√	-	√	√	√	√	√	√	√	√
Bakery by products	√	√	-	√	√	√	-	-	√	√	-	√	√	-	√
Animal fat	√	-	√	√	√	√	√	√	√	√	-	√	√	√	√
Soya oil	√	√	√	-	√	√	-	√	-	√	√	√	-	-	-
Monocalciumphosphate	√	-	-	√	√	√	-	√	-	√	√	-	-	√	-
Manioc	-	√	-	√	-	-	-	-	√	-	-	-	-	-	-
Maize grits	-	√	-	√	√	√	√	√	√	√	-	√	√	√	√
Palm oil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Soya oil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium carbonate	-	-	-	-	-	-	-	-	-	-	√	-	-	-	-
Fish meal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maize gluten feed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Propylenglycol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potato protein	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wheat feed flour	-	-	-	-	√	-	-	-	-	-	-	-	-	-	-

Annex III. Composition of chickenfeeds used in the study.

Sample codes	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Toasted Soyameal															
Palmkernel meal	√	√	-	-	-	√	√	√	-	-	-	√	-	-	√
Cabbage and turnip seeds by products	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Toasted Soyameal	√	-	-	√	√	√	√	√	√	√	-	√	√	-	√
No bitter lupin	√	√	-	√	√	√	-	-	√	√	-	√	√	-	√
Calcium carbonate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beet vinasses	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Cane molasses	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salt	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Magnesium oxide	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Citrus pulp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maize	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rye	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Beet pulp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Soyabean hulls	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potato fruit-juice, concentrated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Liquid lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vegetable fatty acids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wheat semolina	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Triticale	-	√	√	√	-	-	√	√	-	√	√	√	-	-	√
Beet molasses	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coconut, extracted	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maize	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wheat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Barley	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Wheat gluten feed	-	-	-	-	-	-	-	√	-	-	-	-	-	-	-
Garden peas	√	√	√	√	-	√	√	√	-	-	-	√	-	-	-
Sunflower seed, extracted	-	-	-	-	-	-	-	-		-	-	-	-	-	-
Bakery by products	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Animal fat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Soya oil	√	-	-	√	-	√	√	√	-	-	-	√	-	-	-
Monocalciumphosphate	√	√	√	-	√	-	-	-	√	√	√	√	√	√	√
Manioc	-	√	-	√	√	√	√	√	√	√	√	√	√	√	-
Maize grits	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Palm oil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Soya oil	-	√	√	-	√	-	-	-	√	√	√	-	√	√	√
Sodium carbonate	√	√	√	-	√	√	-	-	√	-	√	-	√	√	√
Fish meal	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Maize gluten feed	-	-	-	-	-	-	-	√	-	√	-	-	-	-	-
Propylenglycol	-	√	-	√	-	-	-	-	-	-	-	√	-	-	-
Potato protein	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Wheat feed flour	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Annex IV. ***Feed sample preparation and extraction protocol provided by Neogen.***

1. Prepare the sample extraction solution as indicated below:

   Discard approximately 220 ml fro a 3.8 L container of distilled or deionised water. Using the paper funnel supplied, pour the foil pouch of powdered extraction solvent into the container. To completely dissolve the solution, shake the container vigorously for 2-3 minutes. The solution may appear cloudy when completely dissolved. Store the extraction solution at room temperature.
2. Weigh 10 grams of feed sample (do not grind samples) and place in a jar.
3. Add ~1.5 grams of extraction additive to the jar.
4. Add 75 ml of prepared extraction solution to the jar and swirl to mix.
5. Heat in boiling water bath for 15 minutes.
6. Remove the jar from the water bath. Swril to mix. Do not allow sample to cool prior to testing.
7. Allow sample to settle briefly to allow for a clear layer to form. Low speed centrifugation may be required for more viscous samples.
8. Remove the appropriate number of sample tubes, label and place in tube rack.
9. Using a new transfer pipette for each sample, transfer approximately 0.5 ml of each extract to a sample tube.
10. Place a new test strip with the sample end down into the sample tube.
11. Keeping strip in vertical position, allow the strip to develop for 25 minutes.
12. Visually interpret results as indicated in kit insert as well as measurement of results with Neogen Accuscan detection system is recommended.