EVALUATION OF THE « *MELISA-TEK*^{TM} RUMINANT KIT» (ELISA TECHNOLOGIES, INC., GAINESVILLE, FL, USA) FOR THE DETECTION OF RUMINANT PROTEINS IN PROCESSED ANIMAL PROTEINS

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1. INTRODUCTION

Currently, unless exceptions for fish meal, processed animal proteins (PAPs) including meat and bone meals are banished from use as feed ingredients for all farmed animals. Moreover, the use of PAPs is controlled within the European Union through several regulations which on one hand prohibit explicitly the feeding of mammalian PAPs to ruminants (Regulation EC 999/2001), the feeding of animals with proteins from the same species (Regulation EC 1774/2002) and on the other hand establish 3 categories of animal by-products (ABP) corresponding to different safety levels. In consequence, only material from category 3 which correspond to the lowest risk level could be used to feed farm animals if the ban on use of PAPs is lifted.

Besides the legislation, efficient screening tests as well as validated methods are tremendously needed to detect in routine PAPs as well as to identify their origin at the species level before a reappraisal of the total MBM ban and a possible safe re-introduction of non-ruminant PAP in feed. Among the techniques to aim this goal, immunoassay-based tests are of interest.

According to a risk assessment study conducted by Det Norsk Veritas Ltd. (DNV consulting, 2006), a threshold limit of 2% of ruminant PAP in non-ruminant PAP would still provide a safe level of protection of human and animal health regarding the Bovine Spongiform Encephalopathy (BSE) risk. European Fat Processors and Renderers Association (EFPRA) proposed the re-entry of certain PAPs for use in Feeds (EFPRA, 2006) and more recently the use of non-ruminant PAPs in Feeds for Aquatic Species (Aqua-feeds) (Woodgate, 2007 a) with respect of the legal intra-species ban and the introduction of a tolerance level of 2% of ruminant material.

In a first time, the use of the dipstick test ***"ReVeal® for Ruminant in Feed"*** (Neogen Corporation, Lansing, MI, USA) as screening method for the detection of ruminant PAPs in PAPs was proposed by EFPRA (Woodgate, 2007 b). However, the possibility to have false negative results with samples containing levels as high as 10 % in mass fraction of some ruminant PAP was demonstrated (Fumière *et al.*, 2008). Another screening test, the ***MELISA-TEK™ Ruminant Species Kit*** (ELISA Technologies Inc., Gainesville, FL, USA), in combination with the ***MELISA-TEK™ High Sensitivity Extraction Kit*** (ELISA Technologies Inc., Gainesville, FL, USA) is now proposed by EFPRA. Based on the results of studies conducted by the CCL Nutricontrol (Veghel, the Netherlands) (Vaessen, 2009 a & b), all tested samples containing 2% of ruminant material were detected as positive and only a few samples containing 1% of ruminant material in other PAPs were not detected.

Preliminary tests using only the ***MELISA-TEK™ Ruminant Species Kit*** were already performed by the CRL-AP in 2008 with the conclusion that it could be a good alternative to the Neogen assay for the detection of ruminant PAPs in PAPs when decision criteria proposed by CCL Nutricontrol are used despite restricting aspects of a more skilled staff and specific laboratory equipment (Fumière *et al.*, 2008).

The purposes of the present document are : i) to summarize the available data on the ***MELISA-TEK™ Ruminant Species Kit*** (ELISA Technologies Inc., Gainesville, FL, USA) ; ii) to provide a scientific review of the assessment study conducted by CCL on this kit ; iii) to analyse a limited number of samples in order to check the potential of the method as a first line screening technique with a special focus on the main samples giving false negative results with the Neogen dipstick test.

2. PRINCIPLE OF THE TEST

The ***MELISA-TEK™ Ruminant kit*** (ELISA Technologies Inc., Gainesville, FL, USA) is intended to detect ruminant muscle tissue in extracts made from cooked meat and feed products such as meat meals and meat and bone meals. The assay is a sandwich ELISA based on species specific recognition of troponin I by specific monoclonal antibodies.

Briefly, the test can be described like this : a troponin I specific monoclonal antibody is immobilized to the wells of the test strips, which captures troponin I present in the samples or controls. After a wash step, a second troponin I specific monoclonal antibody, which has been biotinylated, is allowed to bind to the troponin I present in the well. After a second wash step, a streptavidin-horseradish peroxidase (SA-HRP) conjugate is added which binds to the biotinylated secondary antibody, and any unbound SA-HRP is washed away. The tetramethylbenzidin (TMB) substrate is added, which reacts with the HRP of the conjugate, causing a colour change in proportion to the level of troponin I originally bound to the well. Finally, a stop solution is added after a specific time and colour development is evaluated using an ELISA plate reader (reading at the wavelength of 450 nm).

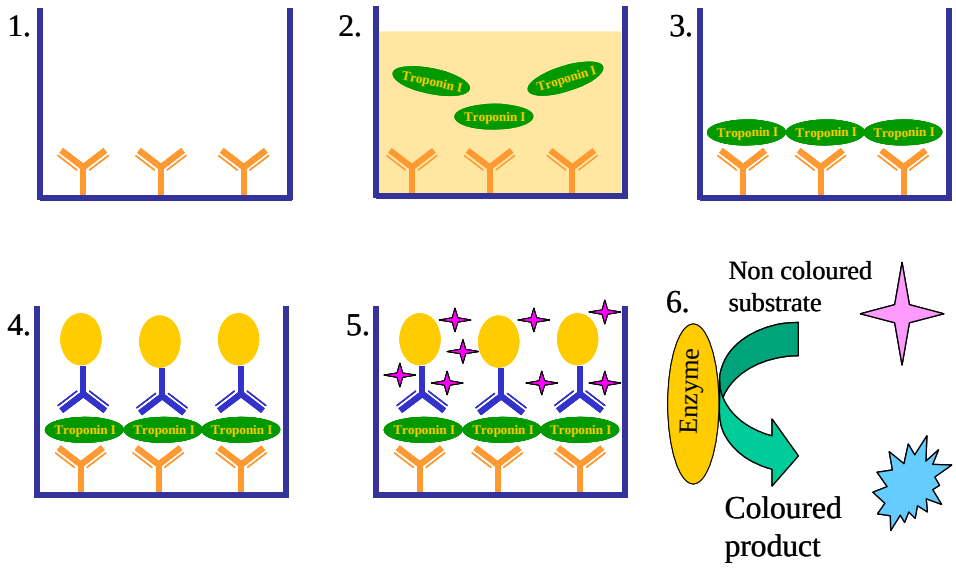

FIGURE 1 : GENERAL SCHEME OF A SANDWICH ELISA

Based on results communicated by the CCL (Vaessen, 2008), it seems that the MELISA-TEK kit could be a good alternative to the Neogen test for the detection of ruminant PAPs.

According to the manual provided by the manufacturer [1], the MELISA-TEK SPECIES kits are able to identify the presence of thermo-stable muscle tissue protein in meat and bone meals and animal feed samples containing muscle tissue at levels of approximately 0.05% or greater. In-house testing by the manufacturer indicates the following detection limits:

1. *Muscle tissue : lean muscle tissue prepared at up to 138 °C and 4 bars pressure during 20 minutes in a closed container is detected at a 1:2000 dilution of an 1:10 extract equivalent to a concentration of 0.005 % lean muscle tissue in a sample.*

2. *Meat and bone meal : a meat and bone meal (provided by IRMM) processed at 133 °C and 3 bars during 20 minutes composed of 50 % beef/50 % pork is detected at a 1:100 dilution of an extract equivalent to a concentration of 0.5 % in weight of meat and bone meal in a sample.*

3. *Animal feed : species thermo-stable muscle tissue protein antigens are detected in animal feeds containing 5 % of a meat and bone meal processed at 133 °C and 3 bars during 20 minutes composed of 50 % beef/50 % pork equivalent to a concentration of 2.5 % in weight of* (ruminant) *meat and bone meal in a feed sample.*

The samples (5g) are extracted according to the kit's manual but an extra concentration step using the ***MELISA-TEK™ High Sensitivity Extraction Kit*** (ELISA Technologies Inc., Gainesville, FL, USA), funnels and concentrator tubes is added to the protocol by the CCL. With this kit, ruminant meat and bone meals can be detected at 0.1% (w/w) in feeds, and ruminant muscle tissue can be detected at 0.01% in solution[2].

Concerning the specificity of the test, numerous organic and inorganic compounds commonly present in animal feeds have been tested and do not interfere with the test [2].

A decision boundary using criteria diverging from the manufacturer's instructions is also proposed by CCL Nutricontrol. It takes the blank-subtracted average OD of the analysed negative samples + 3 times the standard deviation of the OD on these negative samples to set the cut-off value below which a sample is declared negative if all the controls indicate a valid test (Vaessen, 2009 a & b).

[1] ELISA Technologies Inc. MELISA-TEK™ SPECIATION KITS for MEAT & BONE MEALS and ANIMAL FEEDS. Instructions for use. Revision 70308-V1.

[2] ELISA Technologies Inc. Web site. http://www.elisa-tek.com/species%20identification.htm (29/04/2009).

3. STATUS OF THE KNOWLEDGE ON THE PERFORMANCES OF THE MELISA-TEK™ RUMINANT KIT

Except the documentation provided by the manufacturer, the information on the test is quite rare in the literature. The only reference found is an assessment study performed by Myers *et al.* (2007) in which they compared the performance characteristics of two enzyme-linked immunosorbent assay (ELISA) test kits: the MELISA-TEK test (ELISA Technologies) and the BioKit for (Cooked) Species Identification test (Tepnel BioSystems, Stamford, Conn. USA). It should be mentioned that this study assess the potential of the test to detect the presence of ruminant protein in feed and not in other PAPs. The selectivity of the tests was assessed by testing 60 dairy feed samples that contained no added animal proteins. The sensitivity was determined by evaluating 60 samples per level of fortification of the same feed containing levels of bovine MBM (BMBM) comprised between 0.025 and 2%. The BMBM was prepared according a rendering process with temperatures ranging from 125°C to 131°C (during 5 minutes and probably not under pressure like in Europe) for a total processing time of approximately 30 minutes. According to that study, the MELISA-TEK test gave excellent results for the selectivity but failed the sensitivity assessment at the tested levels except at the 2% level. Nevertheless, the test still detected 93% of the 1% level positive samples. In the conclusion of the paper, Myers *et al.* point out two important information : i) among the four different kits studied by the authors in that paper and in another one (Myers et al., 2005), the lateral-flow tests "ReVeal® for Ruminant" provided by Neogen Corporation and the ELISA-based assay "MELISA-TEK™" Ruminant kit from ELISA Technologies all use the same antibodies ; ii) the use of a study-specific criterion for determining true-negative and true-positive results that is different from the instructions of the provider was necessary to correctly assign all the truly negative samples : results below 0.150 OD absorbance units correspond to negative results and above 0.180 OD absorbance units to positive ones.

4. REVIEW OF THE CCL NUTRICONTROL STUDY ON THE PERFORMANCES OF THE MELISA-TEK™ RUMINANT KIT

In the light of the data presented in the paper of Myers *et al.* (2007), it appears very important to check whether the CCL assessment study on the performances of the MELISA-TEK™ Ruminant kit addressed the following questions :

1. Using the same antibodies as the Neogen dipstick tests, does the MELISA-TEK™ present the same drawbacks in terms of possible masking effect and susceptibility to the process conditions?

2. Besides the CCL study, the assessment study of Myers *et al.* (2007) is the only other one in the scientific literature. It was performed only with American samples to detect bovine meat and bone meal **in feed** and the protocol used did not integrate the MELISA-TEK™ High Sensitivity Extraction Kit. Within the protocol used by the CCL, is the test able to detect 2 % of ruminant PAPs in other PAPs?

3. The study of Myers *et al.* underlines problems to the set criteria for assigning negative and positive samples. Is the criterion established and proposed by the CCL as the decision limit usable by other labs ?

4.1. SAMPLE SET

The sample set used in the CCL study is quite large :

- 72 PAPs without any ruminant material (blank samples)
- 8 pure European bovine PAPs heat treated from 133°C to 145°C
- 8 pure European ovine PAPs heat treated from 133°C to 145°C
- 1 bovine spray dried plasma
- 11 commercial mammalian mix samples

All the samples were tested alone or in mixture. An American bovine meat and bone meal giving false negative results with the Neogen strip test even at high level of spiking was provided by the CRL-AP and is integrated in the study. The sample set appears to be as much representative as possible.

4.2. SPECIFICITY

The 72 blank samples (PAPs without any ruminant material) were tested giving only 1 false positive results (Specificity = 99%). The other samples containing ruminant material were also tested pure with no false negative result. Due to the limited available amount of sample, the American bovine PAP was not tested alone.

Based on the results provided by the CCL, no major problem of aspecificity can be identified.

4.3. SENSITIVITY

Hundred sixty three mixes of European PAPs were tested. Ruminant PAPs heat treated at the different temperatures were spiked at levels of 2% or lower in non ruminant PAPs mainly pig PAPs and fish meals. **All the samples containing 2% of ruminant PAPs were detected as positive. The few false negative results were due to samples containing 1% or lower levels of ruminant material.**

Mixes of European ruminant PAPs were also spiked in other PAPs including avian and poultry as well as porcine PAPs or fish meals. **No false positive result was observed.**

Twelve mixes containing the American bovine meat and bone meal at levels of 1 or 2% were also tested. **Only one false negative result was observed with a sample containing 1% of the BMBM.**

The 11 commercial mammalian mix samples were tested in 19 mixes with non-ruminant PAPs. **Only 1 sample containing 1% of ruminant material gave a false negative result.**

Based on the results provided by the CCL, the test appears to be fit to detect 2% of ruminant PAPs in other PAPs whatever its origin or its production process. No problem of masking effect (e.g. by porcine PAPs) is identified at the level of 2%. Below this level, a majority of the false negative results (6/9 false negative results) were obtained with mixes containing 5 different porcine PAPs; the remaining ones (3/9) were observed with mixes containing the same fishmeal but also low levels of the same ruminant material (0.1, 0.2 and 0.5 % respectively).

4.4. SETTING OF A SPECIFIC DECISION CRITERION

The setting of specific decision criteria is crucial for the correct application of the test. These criteria diverge considerably when we consider what is applied by the kit manufacturer, by Myers *et al.* (2007) and by CCL. We ourselves had in fact used a still somewhat different system in the addendum to the study of the assessment of the Neogen dipsticks (Fumière *et al.*, 2008). It was normally based on what CCL had explained to the CRL-AP but in practice the way we used it was slightly different. This latter system should however come up with similar results as CCL because it is clear from the CCL study that OD on blank samples and on negative control samples are almost comparable.

The important point here is to come up with a robust and practical decision system applicable for any laboratory. It should therefore avoid to consider mean OD values on large set of blank samples but just take into consideration the OD figures of the negative control samples (because it is important to subtract these figures from those of the tested sample to avoid working in absolute OD figures).

5. CONCLUSIONS BASED MERELY ON A REVIEW OF THE CCL REPORT

Based on the provided results, the performances of the test at the considered level of contamination are convincing. In conclusion, the CCL assessment study on the use of the MELISA-TEK™ to detect 2% ruminant material in other PAPs appears to be consistent. However, transfer of the technique to another laboratory was not considered in the study. Therefore it seems important to check this on a limited number of samples before giving any final conclusion of the CRL-AP. The decision criteria used by CCL and by us in the addendum to the assessment study of the Neogen dipstick kit (Fumière *et al.*, 2008) should provide identical results. This means that on those samples that were wrongly classified in the addendum of this previous study, better results should have been obtained if the full procedure is followed. If not, it means that there is some transfer problem linked either to the extraction step or to the decision criteria used.

6. EXPERIMENTAL EVALUATION OF THE KIT

The extract of a sample is analysed in duplicate on a plate. The absorption (OD value) is read at 450 nm with an ELISA reader. Average blank OD (blank control OD) is subtracted from the average OD of each sample. In the CCL Nutricontrol study, new decision criteria to classify positive and negative samples are set at an OD of 0.017 (the initial criteria in the kit protocol is an OD 0.100). According to these new decision criteria, a sample is declared as positive if its blank subtracted OD > 0.017 and the controls indicate a valid test. Conversely, a sample is considered negative if the sample blank subtracted OD ≤ 0.017 and the controls indicate a valid test.

The validity of this major modification of the decision criteria has to be checked on a limited number of samples.

6.1. ANALYSIS OF NON RUMINANT PAPS

Twenty-four non ruminant samples (fish meals, pork PAPs and poultry PAPs) available from the CRL sample bank or requested to the CCL were firstly analysed. The reading was made on two ELISA plate readers : one provided by EFFRA (Microplate Reader Model 550, Biorad), the second one from the CRL lab (Novapath™ Microplate Reader, Biorad).

TABLE 1 : RESULTS OF NON RUMINANT SAMPLES OBTAINED WITH A MICROPLATE READER MODEL 550 : ABSORBANCES AT 450 NM OF THE REPLICATES AND THE BLANK CONTROL OF THE PLATE (MEAN OF 2 MEASUREMENTS), BLANK SUBTRACTED OD AND CONCLUSION ACCORDING TO THE CCL CRITERIA

Sample	Description	Absorbance @450 nm OD1	OD2	Mean	Blank OD	OD sample – OD blank	Result OD>0.017
1	Fish meal (DQ/05/0653-01)	0.143	0.136	0.140	0.152	-0.012	Negative
2	Fish meal (DQ/05/0653-04)	0.170	0.136	0.153	0.152	0.002	Negative
3	Fish meal (DQ/05/0653-05)	0.137	0.128	0.133	0.152	-0.019	Negative
4	Fish meal (DQ/05/0653-07)	0.161	0.134	0.148	0.152	-0.004	Negative
5	Fish meal (DQ/05/0653-08)	0.126	0.135	0.131	0.152	-0.021	Negative
6	Fish meal (DQ/05/0653-10)	0.176	0.181	0.179	0.152	0.027	Positive
7	Pork protein 100% (DQ/07/0038-01)	0.329	0.164	0.247	0.160	0.087	Positive
8	Porcine protein 58 100% (DQ/07/0094-04)	0.266	0.143	0.205	0.160	0.045	Positive
9	Porcine protein 40 100% (DQ/07/0094-05)	0.266	0.221	0.244	0.160	0.084	Positive
10	ER22 porcine protein 58 (DQ/07/1089-19)	0.361	0.196	0.279	0.160	0.119	Positive
11	ER7 porcine MBM (DQ/07/1089-20)	0.224	0.255	0.240	0.160	0.080	Positive
12	ER21 porcine meat meal (DQ/07/1089-21)	0.273	0.287	0.280	0.160	0.120	Positive
13	ER12 avian MBM (DQ/07/1089-03)	0.102	0.139	0.121	0.129	-0.009	Negative
14	ER31 poultry MBM (DQ/07/1089-07)	0.133	0.192	0.163	0.129	0.034	Positive
15	ER30 poultry meal standard (DQ/07/1089-10)	0.207	0.150	0.179	0.129	0.050	Positive
16	Feather meal (DQ/07/0142-03)	0.135	0.111	0.123	0.129	-0.006	Negative
17	ER16 avian meal (DQ/07/1089-06)	0.127	0.105	0.116	0.129	-0.013	Negative
18	ER24 porcine/poultry protein 54 (DQ/07/1089-30)	0.113	0.095	0.104	0.129	-0.025	Negative
19	Fish meal (DQ/05/0653-11)	0.131	0.142	0.137	0.127	0.010	Negative
20	Fish meal (DQ/05/0653-13)	0.132	0.147	0.140	0.127	0.013	Negative
21	Porcine protein (DQ/09/0355-04)	0.126	0.139	0.133	0.127	0.006	Negative
22	Pig protein powder (DQ/09/0355-05)	0.132	0.125	0.129	0.127	0.002	Negative
23	ER20 porcine meat meal (DQ/07/1089-23)	0.142	0.134	0.138	0.127	0.012	Negative
24	ER23 porcine protein (DQ/07/1089-26)	0.129	0.132	0.131	0.127	0.004	Negative

Looking at these results on negative samples, different observations can be made:

- Nine samples (samples # 6, 7, 8, 9, 10, 11, 12, 14, 15) on the 24 samples tested give a false positive result.
- Six from these 9 samples (samples # 6, 9, 10, 11, 12, 15) give a false positive result for each of the duplicates. The 3 remaining samples (samples # 7, 8, 14) give a false positive result with only one of the replicate measurements.
- The false positive results are obtained on 3 different plates with controls giving correct results (according to the protocol of the kit) validating the plates.
- The variability of the blank OD in terms of standard deviation is higher than the threshold set by the CCL. The lowest value of the blank OD is 0.115 and the highest value is 0.178.
- No correlation between the false positive results and the blank OD value can be made : two false positive results are obtained on a plate where the blank OD (0.129) is close to the minimum blank OD observed (0.127) whereas the 7 other false positive results are observed on a plate with the highest observed blank OD value (0.160).

The results are globally confirmed when the ODs are measured on a second ELISA plate reader present in the CRL lab.

TABLE 2 : RESULTS OF NON RUMINANT SAMPLES OBTAINED WITH A NOVAPATH™ MICROPLATE READER : ABSORBANCES AT 450 NM OF THE REPLICATES AND THE BLANK CONTROL OF THE PLATE (MEAN OF 2 MEASUREMENTS), BLANK SUBTRACTED OD AND CONCLUSION ACCORDING TO THE CCL CRITERIA

Sample	Description	Absorbance @450 nm OD1	OD2	Mean	Blank OD	OD sample – OD blank	Result OD>0.017
1	Fish meal (DQ/05/0653-01)	0.104	0.095	0.100	0.103	-0.004*	Negative
2	Fish meal (DQ/05/0653-04)	0.126	0.093	0.110	0.103	0.006*	Negative
3	Fish meal (DQ/05/0653-05)	0.090	0.081	0.086	0.103	-0.018*	Negative
4	Fish meal (DQ/05/0653-07)	0.114	0.086	0.100	0.103	-0.003*	Negative
5	Fish meal (DQ/05/0653-08)	0.089	0.091	0.090	0.103	-0.013*	Negative
6	Fish meal (DQ/05/0653-10)	0.123	0.132	0.128	0.103	0.025*	Positive
7	Pork protein 100% (DQ/07/0038-01)	0.383	0.228	0.306	0.191	0.115	Positive
8	Porcine protein 58 100% (DQ/07/0094-04)	0.295	0.173	0.234	0.191	0.043	Positive
9	Porcine protein 40 100% (DQ/07/0094-05)	0.288	0.240	0.264	0.191	0.073	Positive
10	ER22 porcine protein 58 (DQ/07/1089-19)	0.375	0.217	0.296	0.191	0.105	Positive
11	ER7 porcine MBM (DQ/07/1089-20)	0.285	0.286	0.286	0.191	0.095	Positive
12	ER21 porcine meat meal (DQ/07/1089-21)	0.293	0.308	0.301	0.191	0.110	Positive
13	ER12 avian MBM (DQ/07/1089-03)	0.167	0.207	0.187	0.143	0.044	Positive
14	ER31 poultry MBM (DQ/07/1089-07)	0.173	0.224	0.199	0.143	0.056	Positive
15	ER30 poultry meal standard (DQ/07/1089-10)	0.237	0.179	0.208	0.143	0.065	Positive
16	Feather meal (DQ/07/0142-03)	0.164	0.137	0.151	0.143	0.008	Negative
17	ER16 avian meal (DQ/07/1089-06)	0.188	0.148	0.168	0.143	0.025	Positive
18	ER24 porcine/poultry protein 54 (DQ/07/1089-30)	0.142	0.128	0.135	0.143	-0.008	Negative
19	Fish meal (DQ/05/0653-11)	0.190	0.197	0.194	0.135	0.059	Positive
20	Fish meal (DQ/05/0653-13)	0.158	0.172	0.165	0.135	0.030	Positive
21	Porcine protein (DQ/09/0355-04)	0.151	0.159	0.155	0.135	0.020	Positive
22	Pig protein powder (DQ/09/0355-05)	0.152	0.147	0.150	0.135	0.015	Negative
23	ER20 porcine meat meal (DQ/07/1089-23)	0.202	0.159	0.181	0.135	0.046	Positive
24	ER23 porcine protein (DQ/07/1089-26)	0.154	0.149	0.152	0.135	0.017	Negative

*Reading in dual mode (OD value at 450 nm – OD value at 655 nm)

The samples giving false positive results with the first plate reader are still declared positive. Six other samples (samples # 13, 17, 19, 20, 21 and 23) also give a false positive result on the second plate reader. For four of these six samples (samples # 13, 17, 19, 23), both duplicates are positive according to the decision criterion of OD>0.017.

6.2. ANALYSIS OF NON RUMINANT PAPS SPIKED WITH RUMINANT PAPS

The following tables present the results obtained with PAP samples adulterated with bovine PAPs at levels of 1, 2 or 5%. According to samples available, we looked at the mixes giving false negative results at 1% in the CCL study.

TABLE 3 : RESULTS OF NON RUMINANT PAPS SAMPLES ADULTERATED WITH BOVINE PAPS OBTAINED WITH A MICROPLATE READER MODEL 550 : ABSORBANCES AT 450 NM OF THE REPLICATES AND THE BLANK CONTROL OF THE PLATE (MEAN OF AT LEAST 2 MEASUREMENTS), BLANK SUBTRACTED OD AND CONCLUSION ACCORDING TO THE CCL CRITERIA

Description	Absorbance @450 nm OD1	OD2	Mean	Blank OD	OD sample – OD blank	Result OD>0.017
ER22 porcine protein 58 (DQ/07/1089-19) + 1% bovine carcass 145°C (DQ/09/0355-02)	0.133	0.144	0.139	0.166	-0.028	Negative
ER22 porcine protein 58 (DQ/07/1089-19) + 2% bovine carcass 145°C (DQ/09/0355-02)	0.224	0.144	0.184	0.166	0.018	Positive
Porcine muscle141°C (DQ/09/0355-03) + 1% bovine carcass 145°C (DQ/09/0355-02)	0.149	0.142	0.146	0.166	-0.021	Negative
Porcine muscle141°C (DQ/09/0355-03) + 2% bovine carcass 145°C (DQ/09/0355-02)	0.167	0.144	0.156	0.166	-0.011	Negative
Pig protein powder (DQ/09/0355-05) + 1% American cattle PAP (DQ/06/0959-17)	0.163	0.145	0.154	0.166	-0.012	Negative
Pig protein powder (DQ/09/0355-05) + 2% American cattle PAP (DQ/06/0959-17)	0.152	0.150	0.151	0.166	-0.015	Negative
ER22 porcine protein 58 (DQ/07/1089-19) + 5% bovine carcass 145°C (DQ/09/0355-02)	0.237	0.194	0.216	0.154	0.062	Positive
Pig protein powder (DQ/09/0355-05) + 5% American cattle PAP (DQ/06/0959-17)	0.198	0.227	0.213	0.154	0.059	Positive
Pig PAP (DQ/07/0134-09) + 5% American cattle PAP (DQ/06/0959-17)	0.208	0.206	0.207	0.154	0.054	Positive
Pig PAP (DQ/07/0134-09) + 2% (DQ/02/0862-02)	0.241	0.252	0.247	0.154	0.093	Positive
Pig PAP (DQ/07/0134-09) + 2% (DQ/02/1032)	0.591	0.557	0.574	0.154	0.421	Positive
Pig PAP (DQ/07/0134-09) + 2% (DQ/07/0134-03)	0.210	0.188	0.199	0.154	0.046	Positive

Among the samples tested in the CCL study, 5 samples give a false negative result. The mix composed of 98 % in mass of the porcine PAP labelled ER22 porcine protein 58 (DQ/07/1089-19) and 2 % in mass of bovine PAP heat treated at 145 °C (DQ/09/0355-02) is declared positive. Looking at the OD value of the duplicates, only one of the two duplicates gives a positive result. This last observation illustrates once again the variability of the results. It must also be pointed that the porcine PAP used in the mix gives a false positive result when tested alone (see Table 1).

TABLE 4 : RESULTS OF NON RUMINANT PAPS SAMPLES ADULTERATED WITH BOVINE PAPS OBTAINED WITH A NOVAPATH™ MICROPLATE READER : ABSORBANCES AT 450 NM OF THE REPLICATES AND THE BLANK CONTROL OF THE PLATE (MEAN OF AT LEAST 2 MEASUREMENTS), BLANK SUBTRACTED OD AND CONCLUSION ACCORDING TO THE CCL CRITERIA

Description	Absorbance @450 nm OD1	OD2	Mean	Blank OD	OD sample – OD blank	Result OD>0.017
ER22 porcine protein 58 (DQ/07/1089-19) + 1% bovine carcass 145°C (DQ/09/0355-02)	0.200	0.210	0.205	0.180	0.026	Positive
ER22 porcine protein 58 (DQ/07/1089-19) + 2% bovine carcass 145°C (DQ/09/0355-02)	0.258	0.175	0.217	0.180	0.037	Positive
Porcine muscle141°C (DQ/09/0355-03) + 1% bovine carcass 145°C (DQ/09/0355-02)	0.180	0.168	0.174	0.180	-0.006	Negative
Porcine muscle141°C (DQ/09/0355-03) + 2% bovine carcass 145°C (DQ/09/0355-02)	0.194	0.172	0.183	0.180	0.004	Negative
Pig protein powder (DQ/09/0355-05) + 1% American cattle PAP (DQ/06/0959-17)	0.227	0.180	0.204	0.180	0.024	Positive
Pig protein powder (DQ/09/0355-05) + 2% American cattle PAP (DQ/06/0959-17)	0.181	0.175	0.178	0.180	-0.002	Negative
ER22 porcine protein 58 (DQ/07/1089-19) + 5% bovine carcass 145°C (DQ/09/0355-02)	0.295	0.254	0.275	0.170	0.105	Positive
Pig protein powder (DQ/09/0355-05) + 5% American cattle PAP (DQ/06/0959-17)	0.230	0.256	0.243	0.170	0.074	Positive
Pig PAP (DQ/07/0134-09) + 5% American cattle PAP (DQ/06/0959-17)	0.239	0.233	0.236	0.170	0.067	Positive
Pig PAP (DQ/07/0134-09) + 2% (DQ/02/0862-02)	0.266	0.275	0.271	0.170	0.101	Positive
Pig PAP (DQ/07/0134-09) + 2% (DQ/02/1032)	0.635	0.572	0.604	0.170	0.434	Positive
Pig PAP (DQ/07/0134-09) + 2% (DQ/07/0134-03)	0.239	0.211	0.225	0.170	0.056	Positive

There are less false negative results with the second microplate reader. Nevertheless, for the second and the fifth mixes the duplicates present again a high variability and only one of the duplicates is positive. It must also be noticed that all the mixes with 5 % in mass of bovine PAP are detected as positive.

6.3. BACKGROUND NOISE AND REPEATABILITY

From the results presented above and the ones provided by the CCL, we can deduce some considerations :

- The OD values obtained by the CCL for the blank control and for the non ruminant samples are lower (OD ~ 0.080) than these measured by the CRL (OD ~ 0.150). In the experiments conducted by the CRL in 2008 and presented in the addendum of the Neogen kit evaluation (Fumière et al., 2008), the OD values measured were around 0.070.
- The OD values obtained by the CCL seems also much more stable and repeatable than the ones of the CRL. The OD values range observed by the CRL is much larger than the decision criteria of the CCL. In the experiments conducted by the CRL in 2008, the standard deviation observed on the blank control OD values was already of 0.010 and it was generally even higher on the assays performed during this evaluation study. However experts in ELISA do not consider the so obtained variability as abnormal.
- For that reason, the background noise calculated by the CCL to determine the new decision criterion is not transferable because of the too high risk of false results.

In order to identify and explain the problem, additional experiments were done:

1. A plate was performed with 16 replicates of the blank control (no extraction is involved here).
   The OD values measured were comprised between 0.127 and 0.215 (Mean = 0.138 and SD = 0.020) with the Microplate Reader Model 550 and from 0.141 to 0.238 (Mean = 0.169 and SD = 0.023) with the Novapath™ Microplate Reader.
2. A plate with non ruminant samples giving false positive results in previous experiments (Table 1 and 2) was realised. More drastic washing steps were used.

TABLE 5 : RESULTS OF NON RUMINANT SAMPLES OBTAINED WITH A MICROPLATE READER MODEL 550 : ABSORBANCES AT 450 NM OF THE REPLICATES AND THE BLANK CONTROL OF THE PLATE (MEAN OF AT LEAST 2 MEASURMENTS), BLANK SUBTRACTED OD AND CONCLUSION ACCORDING TO THE CCL CRITERIA

Sample	Description	Absorbance @450 nm OD1	OD2	Mean	Blank OD	OD sample – OD blank	Result OD>0.017
4	Fish meal (DQ/05/0653-07)	0.153	0.163	0.158	0.153	0.005	Negative
6	Fish meal (DQ/05/0653-10)	0.145	0.154	0.150	0.153	-0.004	Negative
9	Porcine protein 40 100% (DQ/07/0094-05)	0.153	0.158	0.156	0.153	0.002	Negative
11	ER7 porcine MBM (DQ/07/1089-20)	0.149	0.156	0.153	0.153	-0.001	Negative
13	ER12 avian MBM (DQ/07/1089-03)	0.154	0.172	0.163	0.153	0.010	Negative
14	ER31 poultry MBM (DQ/07/1089-07)	0.156	0.155	0.156	0.153	0.002	Negative

TABLE 6 : RESULTS OF NON RUMINANT SAMPLES OBTAINED WITH A NOVAPATH™ MICROPLATE READER : ABSORBANCES AT 450 NM OF THE REPLICATES AND THE BLANK CONTROL OF THE PLATE (MEAN OF AT LEAST 2 MEAUSREMENTS), BLANK SUBTRACTED OD AND CONCLUSION ACCORDING TO THE CCL CRITERIA

Sample	Description	Absorbance @450 nm OD1	OD2	Mean	Blank OD	OD sample – OD blank	Result OD>0.017
4	Fish meal (DQ/05/0653-07)	0.214	0.220	0.217	0.177	0.040	Positive
6	Fish meal (DQ/05/0653-10)	0.177	0.181	0.179	0.177	0.002	Negative
9	Porcine protein 40 100% (DQ/07/0094-05)	0.184	0.179	0.182	0.177	0.004	Negative
11	ER7 porcine MBM (DQ/07/1089-20)	0.177	0.174	0.176	0.177	-0.002	Negative
13	ER12 avian MBM (DQ/07/1089-03)	0.217	0.199	0.208	0.177	0.031	Positive
14	ER31 poultry MBM (DQ/07/1089-07)	0.182	0.176	0.179	0.177	0.002	Negative

Globally, the results seem to be improved even if the OD values remain high. There is no false positive result when the plate is read on the Microplate Reader Model 550. Nevertheless, 2 false positive results remain when the reading is done with Novapath™ Microplate Reader. For these 2 false positive results, it must be noticed that there is a high variability between the 2 replicates.

3. A plate was performed in another laboratory with high expertise in ELISA technology. The OD values were measured in parallel on the microplate reader of that laboratory and on the Microplate Reader Model 550 giving OD values from 0.201 to 0.258 (Mean = 0.231 and SD = 0.019) and from 0.205 to 0.247 (Mean = 0.228 and SD = 0.014) respectively.

   Remark: The experiment was done under the supervision of a skilled person who did not notice any handling error. The only modification consisted in the bigger volumes of washing buffer used in the washing steps (the volume to use is not mentioned in the kit protocol). Despite this modification, the variability of the blank control OD values remains higher than the one of the CCL.

4. New kits coming from another batch were also ordered to make additional tests.

   4.1. A plate was performed with 16 replicates of the blank control.

The OD values measured were comprised between 0.144 and 0.199 (Mean = 0.177 and SD = 0.021) with the Microplate Reader Model 550 and from 0.149 to 0.240 (Mean = 0.194 and SD = 0.024) with the Novapath™ Microplate Reader.

4.2. A plate was performed with 3 non ruminant samples and 3 mixes containing ruminant PAP. The extract of each sample was tested 5 times.

TABLE 7 : RESULTS OF NON RUMINANT SAMPLES AND SAMPLES CONTAINING RUMINANT PAPS OBTAINED WITH A MICROPLATE READER MODEL 550 : ABSORBANCES AT 450 NM OF THE REPLICATES AND THE BLANK CONTROL OF THE PLATE (MEAN OF SEVERAL MEASURMENTS), BLANK SUBTRACTED OD AND CONCLUSION ACCORDING TO THE CCL CRITERIA

Description	Absorbance @450 nm OD1	OD2	OD3	OD4	OD5	Mean	Blank OD	OD sample – OD blank	Result OD>0.017
Fish meal (DQ/05/0653-01)	0.161	0.156	0.159	0.160	0.143	0.156	0.175	-0.019	Negative
Porcine protein 58 100% (DQ/07/0094-04)	0.153	0.158	0.155	0.149	0.128	0.149	0.175	-0.026	Negative
ER22 porcine protein 58 (DQ/07/1089-19)	0.154	0.175	0.169	0.144	0.111	0.151	0.175	-0.024	Negative
Pig protein powder (DQ/09/0355-05) + 2% American cattle PAP (DQ/06/0959-17)	0.166	0.169	0.167	0.151	0.150	0.161	0.175	-0.014	Negative
Pig PAP (DQ/07/0134-09) + 2% (DQ/02/0862-02)	0.227	0.234	0.239	0.215	0.204	0.224	0.175	0.049	Positive
Pig PAP (DQ/07/0134-09) + 2% (DQ/02/1032)	0.406	0.435	0.427	0.347	0.312	0.385	0.175	0.211	Positive

TABLE 8 : RESULTS OF NON RUMINANT SAMPLES AND SAMPLES CONTAINING RUMINANT PAPS OBTAINED WITH A NOVAPATH™ MICROPLATE READER : ABSORBANCES AT 450 NM OF THE REPLICATES AND THE BLANK CONTROL OF THE PLATE (MEAN OF SEVERAL MEASUREMENTS), BLANK SUBTRACTED OD AND CONCLUSION ACCORDING TO THE CCL CRITERIA

Description	Absorbance @450 nm OD1	OD2	OD3	OD4	OD5	Mean	Blank OD	OD sample – OD blank	Result OD>0.017
Fish meal (DQ/05/0653-01)	0.183	0.178	0.177	0.175	0.160	0.175	0.189	-0.014	Negative
Porcine protein 58 100% (DQ/07/0094-04)	0.188	0.190	0.186	0.181	0.160	0.181	0.189	-0.007	Negative
ER22 porcine protein 58 (DQ/07/1089-19)	0.217	0.237	0.229	0.201	0.178	0.212	0.189	0.024	Positive
Pig protein powder (DQ/09/0355-05) + 2% American cattle PAP (DQ/06/0959-17)	0.198	0.199	0.195	0.181	0.185	0.192	0.189	0.003	Negative
Pig PAP (DQ/07/0134-09) + 2% (DQ/02/0862-02)	0.252	0.254	0.255	0.229	0.223	0.243	0.189	0.054	Positive
Pig PAP (DQ/07/0134-09) + 2% (DQ/02/1032)	0.425	0.456	0.436	0.358	0.330	0.401	0.189	0.213	Positive

Results underlined are OD values of replicates giving a diverging conclusion

The use of new kits does not improve the results:

1. The analysis of blank control replicates shows that the variability of the OD values remains higher than expected. The Standard deviation is > 0.020 (compared to 0.00045 calculated by the CCL on negative samples).
2. False positive and false negative results are still present.

7. FINAL CONCLUSION

Looking at the report of CCL, it seems that the method works pretty well with completely changed decision criteria. When trying to reproduce these results on a limited number of samples, problems of transfer of the method occurred. Negative samples can deliver false positive results but false negative results also appeared for positive samples at 2% of bovine material.

Therefore, the decision criterion set at the OD 0.017 by CCL is probably too strict for an easy transfer of the method. Looking at the variability of the blank control, it appears that the new decision criteria are such that it will lead several laboratories to work within the background noise. Taking into account this problem of transferability, we propose that CCL revises the decision criteria to come with a more robust method for most laboratories or gives a much more detailed protocol on how they work to have such a low variability to be sure that the method will work reliably everywhere. Changing the decision criteria will however imply also to look at the sensitivity of the method with these new interpretation rules and especially if in these conditions it remains possible to reliably detect the 2% level of ruminant material in other PAPs.

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