Chapter 7 : Quantification of PAPs in feed by light microscopy : challenge or illusion ?

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QUANTIFICATION OF PAPS IN FEED BY LIGHT MICROSCOPY : CHALLENGE OR ILLUSION ?

SUMMARY

Among the perspectives of the TSE Roadmap, one is to soften, under well defined conditions, some aspects of the total ban of animal proteins actually in force. One of these aspects is the possibility of introducing a tolerance level on the presence of fishmeal in ruminant feeds as it may originate to side-effect cross contamination from fishmeal containing feeds for non-ruminants. Revising the current feed ban can only start provided adequate control methods are in place for ensuring the correct implementation of a revised feed ban. In this respect, a tolerance level implies a reliable method of quantification for fish meal. The sole quantification method is that proposed by Commission Regulation EC 152/2009 which is the official source to apply for the determination of constituents of animal origin in feed by classical light microscopy. Interlaboratory studies have demonstrated some shortages of the quantification method as stated by the directive, and in some cases illustrated its inapplicability. The present chapter aims at presenting the current situation of the quantification method and its shortages and at focusing on potential improvements of the current EC 152/2009 regulation in this matter. Tracks for an optimization of the quantification are developed and commented. Alternative protocols, such as one using classical microscopy but considering only the bone fraction in a feed, under development are also considered and presented. Perspectives but also encountered difficulties of implementation are discussed.

Keywords: Quantification; Microscopy; Meat and bone meal; Fishmeal; Animal proteins; Feed

7.1. INTRODUCTION

The drastic decrease of positive cases of BSE within the European Union results from the risk reduction measures taken by the European Commission: the total feed ban. As an effect of this positive trend, a checklist for the transmissible spongiform encephalopathies (TSE) was adopted by the European Commission. The document also referred as The TSE Roadmap (2005), proposes some amendments on the total feed ban while guaranteeing consumer protection and high quality food production.

A first amendment was published in Commission Regulation EC 1292/2005 after the detection of bone spicules in tuber and root crops such as beet pulp. The likely origin is bone fragments of wild animals in soil which stick to the beets and end up in sugar beet pulp fed to ruminants. Member states are asked to make a risk assessment before considering a breach of the feed ban.

Another proposed amendment is the possibility of softening the ongoing ban on processed animal proteins under strictly defined conditions. One possibility would be the reintroduction of fishmeal in ruminant feeds at a given tolerance level. A first step in this direction was made in September 2008 by allowing again the utilization of fishmeal in the production of artificial milks (or milk replacers) for young ruminants (Commission Regulation EC 956/2008). Notwithstanding the positive risk assessment of this modification on the total feedban, any future reintroduction of fishmeal in feed would require evaluation of the added amounts in order to verify if thresholds are effectively respected or not. Therefore reliable, i.e. robust and validated, quantification method is a prerequisite. At this moment, the quantification is under study and further work is necessary.

Further improvement in differentiating animal proteins specific to certain species may result in an amendment of the provisions with regard to the use of animal products in feedingstuffs, in particular non-ruminant proteins taking into account the prohibition on intra-species recycling in Commission Regulation EC 1774/2002 – such as poultry meat and bone meal (MBM) to pigs. The introduction of a tolerance level with regard to a small presence of processed animal proteins (PAPs) in feed may be proposed in expectation of discriminatory tests.

7.2. LIMITATION OF THE CURRENT LEGAL QUANTIFICATION METHOD

After the ban of the use of animal proteins for ruminant feed in 1994, analytical methods for the identification and quantification of these animal proteins were needed. The only method authorized for official controls in the EU described under point 7 of Annex VI of recent regulation 152/2009 (applicable from 26th September 2009) is based on light microscopy and contains a quantification protocol that was already published in the annex of Commission Directive 98/88/EC.

According to regulation 152/2009 quantification of PAPs in feed is optional at present only where official analyses need to refer to any estimation of the amount of animal constituents. Regulation indicates that quantification can only be carried out if the constituents of animal origin contain bone fragments which concentrate in the sediment obtained from a settling by tetrachloroethylene. This implies that

adulteration of feed with PAPs lacking any bony structure, as it is the case for soft tissues meals for instance, can not be evaluated.

The described formula used for the estimation of PAPs is:

$$\frac{S \times c}{W \times f} \times 100$$

The parameters are the sediment weight *(S)* at 0.001g, the weight of the sample material used for the sedimentation *(W)* at 0.01g, a correction factor for the estimation of the portion of terrestrial bones in the sediment *(c)* – replaced in case of fish by a correction factor of the portion of fish bones and scale fragments in the sediment (*d)* – and a correction factor for the proportion of bones in the constituents of animal origin *(f)* in the sample examined.

The first cooperative study that included the quantification of MBM was published in September 1998 (De Poorter, 1998). In this study 3 different matrices spiked with high levels of animal proteins, including fish meal,ranging from 2 to 6% were analysed by 25 participants After this study, several qualitative studies on the presence of fish meal and MBM have been organised. From a qualitative point of view, these studies have shown that the most critical mixtures are those containing high levels of fish meals and traces of meat and bone meal. As the laboratory performance of the microscopic method improved, better sensitivities were obtained (Veys & Baeten, 2007, Veys & Baeten, 2008)

Until recently no extended study on the robustness of the quantification protocol have been carried out: the optional character of the method as well as the total feed ban policy, excluding any tolerance or threshold value, probably accounts for this situation. From the few data available those from the STRATFEED project where van Raamsdonk *et al.* (2005), based on quantification results from 6 labs on a set of 10 collection samples, concluded that calculations made according this semi-quantitative method are unreliable or scientifically impossible. Their verdict relied on the fact that *f* can never securely be estimated as also reported by von Holst *et al.* (2006) who on the whole considered the quantification as almost impossible due to a lack of information on the type of PAPs being detected within a blind sample. Indeed the regulation text mentions values for *f* varying according the type of animal constituent from 20% to 60% in case of terrestrial animal meals, and from 10% to 20% in case of fish meals. Regulation also makes the assumption that if the type of animal meal present in the sample is known it is then possible, by applying the adequate value of *f* within the formula, to estimate the final content of animal ingredients in the sample. This assumption is erroneous as this situation never occurs in daily routine.

In 2006, a first interlaboratory study conducted by the Community Reference Laboratory for Animal Proteins in feedstuffs (CRL-AP) had as objective the performance assessment of this quantification method (Veys & Baeten, 2007). The study, referred as CRL-AP ILS 2006, involved 22 participating laboratories from the NRL-AP network each of them being asked to realize 10 quantifications for fishmeal (five duplicated feed samples adulterated at percentages from 0.25% to 1.5%). Results of the study not only demonstrated the shortcomings of the official quantification method, but also illustrated its inapplicability (five participants out of 22 were unable to use the method). The absence of any explanation on how to calculate the *d* factor in the official EU guidelines explained the method inapplicability for the five participants. Quantitative results made by the remaining 17 participants showed nevertheless appealing results. All final estimations of the content of fish in feeds were slightly overestimated. The repeatability or within-laboratory variability between measurements was satisfying with a RSD_r ranging from 12-30%. Contrarily the reproducibility or inter-laboratory variability revealed to be extremely variable with RSD_R ranging from 85-116%. This variability was also independent from the percentage of fish meal adulteration. Considering the formula, investigations on the possible causes of the poor reproducibility focussed on the sedimentation process itself, as the counting of particles is made on the sediment, the eventual empirical choice of *f* value and the way *d* was estimated. Results lead Veys & Baeten (2007) to conclude that the sedimentation process, analysed by the S/W ratio which reflects the proportion of sediment being recovered, was unlikely the major cause for variability. By substituting the different values of *f* chosen by the 17 participants by a unique one, the variability was still present with values of RSD_R ranging from 68-101%. So the impact of the *f* factor was also reduced on the reproducibility which was still not satisfactory. Authors of the study thus made the assumption that the major source of variation was likely the *d* factor or more precisely the manner this factor was evaluated as the official method gives no key to calculate it. The fact that actually only the estimation of *d* relies on the operator skills to differentiate between fish particles and particles of other nature argues in this way too. Conclusions of this first study on quantification were that the quantification method needed major enhancements as it could not reliably be implemented.

7.3. PROPOSED ENHANCEMENTS AND IMPLEMENTATION RESULTS

Based on the conclusions of the CRL-AP ILS 2006 proposals for the improvement of the method were made related to the need of clarifying the way the *c* and *d* factor could be computed as there is no definition. These factors have to reflect the amount of a given type of bone particles over

others within sediments, both being identified by light microscopy. Hence volumes of the particles have to be considered rather than their sole numbers. Volume estimation of different particles over each others can be estimated by point counting (figure 7.1) (Howard & Reed, 2005; Russ, 2005), a stereology derived application realized by applying a square mesh grid engraved into a reticle adapted into one of the microscope's eyepiece. The type of grid chosen was the Counting Pattern NG14 (Pyser-SGI Ltd, Edenbridge, UK) which has 10x10 crosses in a 100 µm mesh over an area of 1 mm².

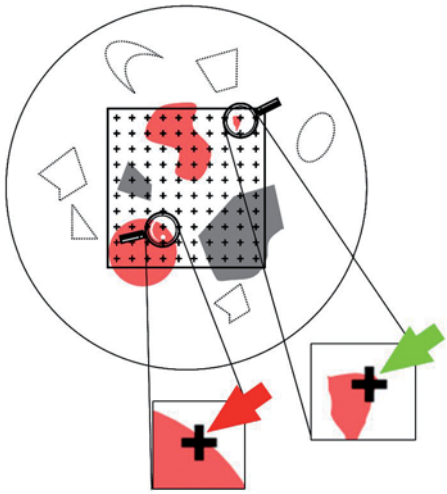

Figure 7.1. Grid counting principle (adapted from Veys & Baeten, 2010). (Red shapes: bone particles, grey shapes: other particles) Doted lined particles outside the grid area not considered for counting. Only particles hit by a cross are counted. Enlarged views illustrate the point counting principle: to each cross a zero-dimension point needs to be associated in order to avoid overestimations (e.g. systematically the upper right corner of a cross). When the zero-dimension point hits a particle it is counted (green arrow), otherwise particles are not counted (red arrow).

For facilitating the discrimination of bone particles versus other it was chosen to work only on sediment stained with Alizarin Red (AR) which preferably colors the hydroxyapatite from the bones in red. Based on the number of respective point counts of each particle type, formulae for estimating *c* or *d* factors were established:

$$c = \frac{Tc}{Tc + Fc + Oc}$$

$$d = \frac{Fc}{Tc + Fc + Oc}$$

Where *Tc* is the number of terrestrial particle counts, *Fc* is the number of fish particle counts and *Oc* the number of counts for particles of other nature. The final estimation of PAPs is then calculated by the modified formula $\frac{Ss \times c}{W \times f} \times 100$ including the AR stained sediment weight (*Ss*) at 0.001g. As they can not be deduced from real blind samples, values of *f* were fixed to 0.40 for terrestrial meat and bone meals (MBMs) and to 0.10 for fish meals. These fixed values were proposed by the European network of National Reference Laboratories (1st CRL-AP Workshop, April 2007, Gembloux, Belgium). This new protocol was tested internally at the CRL-AP on both terrestrial and fish adulterated feed materials by two operators. The initial tests of the new method delivered promising results (Veys & Baeten, 2010 in press): overestimations were no longer observed, the repeatability as well as the intralaboratory reproducibility measure between operators were satisfying.

In order to validate the new protocol a collaborative study was organised in 2007 by the CRL-AP (Veys & Baeten, 2008). The study referred as CRL-AP ILS 2007 had 22 participants from the NRL-AP network who had to realise 10 quantifications for fishmeal (five duplicated feed samples adulterated at percentages from 0.15% to 1%). To be sure that all participants would follow exactly the protocol, strict instructions with fully detailed recommendations (e.g. point counting process as shown in figure 7.1) were communicated. In addition participants had to use a calculation file, with an already encoded default $f = 0.10$, dedicated to the exercise for eliminating possible computation errors. So sedimentation had to be obtained from $W = 10$g (at 0.01g) and sediments stained with AR. Grid counting had to be performed on a well defined number of slides and number of randomly chosen fields per slides. Recommendations were also delivered for obtaining slides with enough particle counts to guarantee a counting precision of ca. 96%.

All participants were this time able to apply the protocol indicating a well defined counting process and *d* factor. Compared to CRL-AP 2006, the repeatability for the results was acceptable and comparable to former study. Relevant improvement of the reproducibility was noted (RSD_R ranging from 50-84%) which is still not enough for validation. Finally Veys & Baeten (2008) observed that quantitative results were still overestimated. This trend to overestimation was even more obvious. Further statistical analyses not only demonstrated that the combined impact of sedimentation process and staining was minor on the quantification variability, but also proved a straight major impact of *d* on the final estimation of PAP content (Pearson correlation coefficient $\rho = 0.809$) and on the variability among the observed values of percentage. Under the fixed conditions of the studies, no other explanation than an overrated value of *d* could account for the observed overestimations of the

values. Therefore Veys & Baeten (2008) conclude that the way *d* is determined is crucial for understanding the variations observed. According to the authors the parameters that actually could affect the determination of *d* are a potential misinterpretation of some AR stained particles described as bones, the number of slides and fields to observe, the slide heterogeneity, the human skills and experiences.

Testing the impact of AR on erroneous characterisations of some particles as bones leading to an overestimation of *d* is relevant as laboratories often do not use the dye during analysis. The arguments put forward for not staining are primarily time savings and the fact that through the multiple staining steps a portion of the sediment is lost – this loss may goes up to 40% of material waste depending a.o. on the amount of hydrosoluble mineral particles within the matrix. Furthermore some advise that AR staining concentrates the bones within the remaining sediment thus possibly leading to a consequent overestimation of *d*. Therefore all participants of the CRL-AP ILS 2007 were asked to realise quantifications again on some samples from the former test (0.4% and 1% fish in duplicate) exactly as they performed during CRL-AP ILS 2007 but without staining of the sediment. Results are summarised in table 7.1.

Table 7.1. Quantification results from 2007 and 2008 experiments

	0.4% Fish			1% Fish		
	CRL-AP ILS 2007	**Unstained 2008**	**Permanent slides 2008**	**CRL-AP ILS 2007**	**Unstained 2008**	**Permanent slides 2008**
Average	1.03	0.78	0.46	1.83	1.47	0.97
STD	0.15	0.12	0.07	0.27	0.17	0.10
s_r	0.43	0.42	-	0.50	0.37	-
RSD_r	42	54	-	27	25	-
s_R	0.72	0.52	0.33	1.28	0.74	0.46
RSD_R	70	67	73	70	50	47

all data are expressed in pourcentage (%)

Average	*Robust mean of all submitted results*
STD	*Standard deviation of the average, calculated from the reproducibility standard deviation devided by the square root of the number of laboratories*
s_r	*Repeatability standard deviation (within laboratory variability)*
RSD_r	*Relative repeatability standard deviation*
s_R	*Reproducibility standard deviation (within plus between laboratory variability)*
RSD_R	*Relative reproducibility standard deviation*

Although estimated amounts of fish were this time slightly closer to the expected values the overestimation was still noted. Values of repeatability and reproducibility were not relevantly improved and remained unsatisfying. Thus staining or not the sediment did not influence on the quantification. Consequently AR staining, although being optional, can only be recommended as not only facilitates the distinction of bones, fish bones and fish scales particles vs. others but also "purifies" the sediment from numerous particles having poor identification values for quantification.

Concerning an optimal number of slides and fields to analyse prior to determining *d*, proposal on a minimum amount of particles to count has already been done for CRL-AP ILS 2007 study. A counting precision of ca. 96% can already be achieved by realising the counting exercise on 20 fields (5 random fields on 4 slides) provided the mean number of counts per field is ≥25. Under the same condition a higher precision, such as 98%, would involve to count 100 fields (5 random fields on 20 slides or 10 random fields on 10 slides). This however would require too much time – furthermore often not enough sediment material is available for preparing so many slides. So a limited effort in order to reach a counting precision of 95% is enough instead of fastidious counting for a very limited gain of precision. Instead more attention should be paid on the sub-portion of the sediment used for the counting. Actually operators should insure that prepared slides should really reflect the sediment homogeneity or relative composition. Therefore the utmost care for preparing slides should be taken when subsampling sediment with a spatula for making slides, or in other terms avoiding slide heterogeneity. In this way for insuring independence of sample probing as mentioned by Howard & Reed (2005), it might be recommended to observe few fields (e.g. 5) on more slides than a large number of fields (e.g. >10) on a reduced number of slides – except when all the sediment is spread on a single or two slides. The quality of slide is also critical for determining *d*. Slides should not have an overload of particles: an excessive density of particles (mean number of counts/field >50) leads to difficult grid counting for the operator's eyes. Large amounts of particles may cause particles overlap which could affect accurate *d* estimation as some particles are hidden. Therefore sieving of the sediment with a 250µm square mesh sieve to obtain a coarse and a fine sediment fraction is advised. Of note sieving has to be realised on well adapted sieve's size to avoid possible loss of material and on perfectly cleaned sieves in order to prevent from cross-contaminations. Different slides can then be prepared from these two fractions to guarantee that no major overlap of particles would occur. Finally, in order to verify the influence of slide preparation on the determination of *d*, the following test was conducted. The 22 participants to the previous CRL-AP ILS 2007 were asked to quantify PAPs on two blind sets of 6 permanent slides. Slides were prepared from AR stained sediments of the formerly used 0.4% and 1% fish adulterated samples. The sets of slides were prepared at the CRL-AP and homogeneity among slides was checked. The two sets were sent successively from one participant to the other so that all measures were made on exactly the same particles. By doing so the source of variability was reduced to the randomization for the field selection (5 fields/slide) and the operator capability of differentiating fish particles from others. Results are shown in table 7.1. The computed values of PAPs were no longer overestimated (0.46% for the expected 0.4% and 0.97% for the expected 1%) but the reproducibility was not significantly improved. This indicates that the accuracy of the quantification method is linked to the quality of slide preparation. Slides must be homogenous regarding the sediment composition. The lack of reproducibility improvement even through this test might logically be related to the microscopist's ability to discriminate correctly particles.

It is known that fish particles have a great morphological variability: fish bones, cartilage fragments, skull chondroid bones fragments, gills, fish scales, otoliths and denticles have specific criteria of identification. Some small particles although originating from fish, may not be defined as fish because of the absence of some markers or their versatile staining reaction with AR as for chondroid particles. It might therefore be supposed that if the same exercise would be realised on terrestrial PAPs adulterated feed, the reproducibility could be expected to be better. But considering human skill implies also a correct use of the microscope as it can lead to erroneous estimations: correct Köhler illumination and sharp focussing is required for a correct grid counting. Effectively bad focussing can lead to over-projection of the particle margins; it is known that over-projection generates overestimations (Howard & Reed, 2005). From those data it appears that the correct identification of particles, animal *vs.* other nature, is the keystone for the quantification method, other identification ways less subject to human interpretation (e.g. NIR microscopy) could be very valuable alternative approaches for the quantification of PAPs in feed.

7.4. DEVELOPMENT OF ALTERNATIVE METHODS

From reflections on the CRL-AP ILS 2007 results, the Danish Plant Directorate proposed in 2008 to develop an alternative approach for quantifying PAPs in feed by light microscopy. The principle of the alternative method is also based on grid counting of particles on slides prepared from AR stained sediments. This method has until now only been tested in a minor scale but has shown results promising enough to be mentioned. Basically the method differ from the former as it bases on standard counts for MBMs and fish meals in a feed

spiked with 0.1% of MBM and fish meal respectively. These standard counts express the total grid counts if all animal particles were counted in a feed with 0.1% of MBM or fish meal respectively. Applied on blind samples, where MBM or fish meal has been identified, an evaluation of the MBM or fish meal particles is made by grid counting on the slides until at least half of the standard counts are reached. Finally final quantification is performed by a calculation integrating different basic factors such as the sediment weights, number of counts in a part of sediment, the standard counts etc.

Standard counts ($CO_{0.1}$) are established as follows. From a standard compound feed adulterated at 0,1% with a standard MBM (f = 0.54) an AR stained sediment is produced from 10g sample accordingly the protocol described in Annex VI of regulation 152/2009. The whole sediment is mounted on slides and all MBM particles (bones, cartilage…) are counted by using the grid counting protocol described previously. The number of counts achieved expresses the standard count ($CO_{0.1}$) in a sample having 0.1% of MBM. The same procedure is followed to establish a standard count for fish meal in a sample adulterated at 0.1% with a fish meal (f = 0.12).

The following standard counts for MBM and FM have been experimentally established.

- $CO_{0.1}$ for MBM (f = 0.54) = 1600 counts
- $CO_{0.1}$ for fishmeal (f = 0.12) = 690 counts

These standard counts have been tested with samples from previous CRL-AP proficiency tests and satisfactory results were reached.

Once the standard counts are determined, quantifications of MBM and fishmeal from blind samples are realized according the following procedure. An AR stained sediment is produced from 10g sample accordingly the protocol described in Annex VI of regulation 152/2009. The weight *Ss* is recorded at 0.001 g. A representative amount of the stained sediment is transferred to a hollow slide. Mounting medium used is glycerol. All animal particles from terrestrial/fish are counted from the slide by grid counting. The counting proceeds until half of the standard counts ($CO_{0.1}$ / 2) for the given animal ingredient in question terrestrial or fish is reached (i.e. at least 800 counts for MBM and 345 counts for fishmeal). When this value of $CO_{0.1}$ / 2 is reached counting continues until the slide in work is ended. The value of counting reached is referred as sample count (CO_s). The remaining AR stained sediment (Ss_r) is weighed at 0.001 g. The weight of the portion of the sediment used for counting (S_c) is calculated $S_c = Ss - Ss_r$. Then the total count (CO), representing the total counts if the whole sediment was counted, is calculated $CO = Ss/S_c \times CO_s$. Eventually the final estimation of PAPs is evaluated by the formula $CO/CO_{0.1} \times 0.1$ using the respective $CO_{0.1}$ values for MBM or fishmeal. The equation on total is expressed as:

$$\frac{Ss \times CO_s}{(Ss - Ss_r) \times CO_{0.1}} \times 0.1$$

Results achieved by the Danish Plant Directorate team on 3 samples prepared by CRL-AP adulterated at 0.15% Fish, 0.4% Fish and 0.1% MBM are presented in table 7.2. They are close to the expected values and therefore promising.

Table 7.2. Preliminary quantifications with alternative method

Samples	CO_s	Ss (mg)	Ss_r (mg)	Sc (mg)	CO ($Ss/Sc \times CO_s$)	% of PAP ($CO/CO_{0.1} \times 0.1$)
Test $CO_{0.1}$ for fishmeal = 690						
0.15% Fish	397	179	112	67	1060	0.15
0.4% Fish	462	179	151	28	2953	0.43
Test $CO_{0.1}$ for MBM = 1600						
0.1% MBM	909	87	38	49	1613	0.10

CO_s *Sample count*

Ss *Stained sediment weight*

Ss_r *Remaining stained sediment weight*

Sc *Portion of stained sediment used for counting*

CO *Total count*

$CO_{0.1}$ *Standard count*

7.5. CONCLUSIONS AND PERSPECTIVES

Both enhanced official method and alternative quantification method present advantages and drawbacks which are presented in table 7.3. Aside the benefits and shortcomings of each method, some common features are interpellant. The weakness for both microscopic methods of quantification is that both are applied only on the sediment and thus base on the sole detection of particles of high density (bones…) which represent only one fraction of PAPs. None of both methods is thus able to consider the entire PAPs for the quantification. Consequently the impact of the *f* factor still influences on the final estimation provided it is not fixed. Fixing values of *f* is therefore a prerequisite for further method improvement and validation. However the way *f* factors would be fixed should be decided with view to "worst case" scenarios – i.e. considering the variability of bone content among different MBMs and fish meals.

Table 7.3. Comparative advantages and disadvantage of both quantification methods

Enhanced quantification method		**Danish quantification method**	
Advantages	**Drawbacks / Risks**	**Advantages**	**Drawbacks / Risks**
Estimate animal particles over other types of particles	Slide preparation is critical (density of particles must be high enough for accuracy of counting and spreading of particles must be homogenous)	Count only animal particles	Still a fixed f-factor for $CO_{0.1}$ (if the f-factor differs much it influence the calculated quantification)
Method is fast (restricted number of fields per slides)	Field selection must be randomized	Do not sieve sediment (minimize the influence of different particle volume)	At high contents of animal ingredients perhaps only one slide is needed to be counted (inhomogeneous sediments influence much)
Repeatability is satisfying	Overestimation source is still to elucidate	Counting all animal particles in the slide (not choosing a reduced number of fields)	Fish products with high content of scales may cause over estimation
	Rely on operator's identification skills	Overestimation is only a small risk	Working with hollow slides may cause masking of some animal particles (overlays)
	Reproducibility still to be improved	Once $CO_{0.1}$ calculated, only minimum of counts à faster à more relaxing to eyes	If counts of animal particles in slides differs much – one may add counts from some more slides
			Repeatability and reproducibility not yet tested
			Time consuming estimation of $CO_{0.1}$
Advantages common to both methods		**Drawbacks / risks common to both methods**	
Alizarin Red staining concentrates bones in sediment and eliminates other types of particles		Consider only animal particles present in sediment (bone, cartilage, otoliths, scales…)	
Grid counting considers volumes		Homogeneity of sub-samplings taken from stained sediment for slide preparation must be insured	

Both microscopic quantification methods strikingly emphasizes on the need of representative slide preparation and sediment sub-sampling as it is proven to play a significant impact on the final estimation of the PAP amount. At writing time, complementary series of tests allying standardized slide preparation and influence of identification skills of the operators are undertaken in order to verify how the reproducibility can be improved by this way. This holds true for both methods. Simultaneously, the alternative quantification method is being tested by other labs and a next coming great scale interlaboratory study is yet planned for evaluation of the method repeatability and reproducibility. Notwithstanding real possibilities of improvement for both light microscopy based quantification methods, it must be emphasized improvements will be relevant to the accuracy rather than to the precision (reproducibility). This assumption can readily be predicted from the origin of the quantification method by light microscopy which is the verification of declaration of feed. These methods, still in use, are effective for estimating ranges of percentages (0-5%, 5-10%, 10-50%) rather than a precise percentage *in se*. Furthermore, for quantification of PAPs in feed, authorities focus on determining amounts at levels ranging from 1% to 0.1%, the latest being officially considered as the limit of detection by microscopy. Therefore using microscopic semi-quantitative methods, because based on identification skills, would imply to accept broad intervals of confidence around estimated values of animal ingredients percentages in feed. Real quantitative methods should rely on other scientific approaches which are presented now.

Both microscopic methods rely on grid counting for taking into account the size of the particles rather than their numbers. Interestingly the same concept can be transposed for quantification of PAPs by NIRM (cf. Fumière et al. (2009) for an overview of NIRM principle and use in PAPs detection) by a mapping matrix which allows limiting the number of spectra to a randomized number of hits on a single layer of feed particles selected by a grid pattern. The utilization of NIRM has one striking advantage over the presented methods: it allows getting free from the human identification factor. However the NIRM approach will face the same concerns on the homogeneity of sub-sampling fractions taken for the analysis –either analysis is made from the pure feed or from the sediment. At time being the NIRM option for quantification is still at its preliminary stage but need to be consider as a real alternative solution as up to now no other methods related to detection of PAP in feed are suitable for quantification purposes. Effectively, quantification methods based on real-time PCR or immunoassay are unrealistic. Real-time PCR kinetics express numbers of copies of animal DNA sequences detected in a feed and hence quantifies this initial number of target copies. However in PAPs there is no link at all between the amount of PAP present in a feed, expressed in mass fraction, and the number of DNA target copies as commented by Fumière et al. (2009). Immunochemical test reactions on PAPs detection are too versatile not only in intensity but also in sensitivity and specificity that could be improved. Therefore immunoassay reading parameter must be considered as unsuitable for quantification purposes (Fumière et al., 2009) up to now.

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