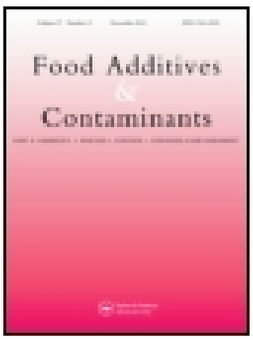

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Collaborative study on the effect of grinding on the detection of bones from processed animal proteins in feed by light microscopy

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ABSTRACT

Bone fragments are essential structures for the detection of processed animal proteins (PAPs) in feed by light microscopy for official controls according to Annex VI of European Union Regulation EC/152/2009. The preparation of samples submitted for analysis requires a grinding step to make them suitable for microscopic slide preparation and observation. However, there are no technical guidelines set down for this step despite the fact that it can lead to an increase in bone numbers due to fragmentation. This was demonstrated by an in-house study carried out by the Irish National Reference Laboratory (NRL) for animal protein detection. The present collaborative study investigated the possible effects of three different grinding conditions on the final result for a feed adulterated with 0.05 and 0.01% (w/w) of PAP. The microscopic analysis either combined or not with an Alizarin Red staining was carried out by 10 different laboratories. The results demonstrated that although a large variation in the numbers of bone fragments was noted, five of the six different grinding/staining combinations applied at two levels of PAP adulteration did not significantly (at $p = 0.05$) differ from one another. The only exception occurred when grinding the feed containing 0.05% of PAP with a rotor mill equipped with a 0.5-mm sieve and combined with a staining which resulted in a greater number of bone fragments by forced fragmentation. Overall, the impact of the grinding/staining combinations on the final results was shown to be negligible when considering the regulatory limit of detection (LOD) requirement for the method and the current rules of implementation of the light microscopic method. From a total of 180 analyses carried out on the feed matrix containing 0.05% of PAP no false-negative result was observed, and at a level of 0.01% PAP only 10 false-negative results occurred.

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Processed animal proteins; light microscopy; grinding; feed

Introduction

Concerning the detection of processed animal proteins (PAPs) in feed by official methods, bones are key structures for disclosing the presence of terrestrial PAPs versus fish PAPs by light microscopy according to Annex VI of European Union (EU) Regulation EC/152/2009 (EU 2009). According to this regulation, amended in 2013 by EU regulation EC/51/2013 (EU 2013), an average pivot value of five fragments regardless of size (but generally in a range between 50 and 500 μm) of a given nature (terrestrial or fish) found through the observation protocol imposes one to declare the sample as positive for this nature if above this value. Grinding of feed possibly containing traces of such PAPs could therefore result in the multiplication of a low number of bones due to fragmentation, and possibly lead to a positive result. In 2014, based on this hypothesis, an in-house study on the effect of grinding on bone spicule fragmentation was conducted by the Irish National Reference Laboratory (NRL) for animal protein detection. The results of this unpublished study were presented at the annual

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workshop of the EU Reference Laboratory for the detection of animal proteins in feedingstuffs (EURL-AP) in Latvia in 2014. Among the conclusions, the study provided evidence that grinding increases the number of bone fragments and that the most severe effects likely occur after the few first seconds of grinding, making it unavoidable. Assumptions about the influence of the type of grinder as well as the hardness of the matrix material on the fragmentation were also put forward for discussion. Therefore, there is a risk that a non-harmonised analytical approach might result in different qualitative results if some grinding devices lead to a much higher multiplication of bone fragments for a given feed matrix. This risk is also real because the grinding of a starting amount of 50 g of sample is commonly required due to the heterogeneity of the feed. Furthermore, the legal text only specifies the use of grinding equipment (mill or even mortar) without any other technical recommendation on the type of equipment (EU 2009, 2013).

Although this first in-house study led to interesting conclusions, those were only preliminary ones and their possible implications to the analysis of PAPs in feedingstuffs could not be addressed. Actually to meet its objective, the Irish study of 2014 was based on an artificial adulteration model: bone fragments were added at a concentration of one bone per g of matrix. This synthetic approach leads to an adulteration level that cannot be expressed as a mass fraction, thus this model is not similar to adulteration by PAPs comprising soft tissues, such as muscles, and only a proportion of bones which may vary – generally from 10% to 60% according the type of animal constituent (Veys et al. 2012). Furthermore, the entire sediment was observed on purpose to survey the fragmentation of the bone spicules, which was the object of the study. In real-world practice, however, the entire sediment is rarely fully analysed. Only a fraction of it is used, which is legally limited to the amount required to prepare a maximum of four slides from the total sediment. In order to improve recovery and isolation of the bone fragments, only Alizarin Red-stained bones were used in the synthetic adulteration during the study. This staining is known to decrease the amount of sediment while concentrating bones (Veys et al. 2012). In absence of literature on the subject, the findings of the Irish study in 2014 triggered the present study. The objective was to investigate further whether different grinding procedures and equipment, combined or not with Alizarin Red staining, applied by different laboratories can influence the analysis of a given feed matrix contaminated with PAPs at concentrations below 0.1% (w/w), which is the level that the method must be able to detect according to the legal requirement.

Materials and methods

Grinding parameters

The possible influence of the type of grinder was investigated. Prior to the study preparation, a survey was conducted among the NRLs to define which type of grinding equipment was the most used among laboratories. Fourteen NRLs replied to this enquiry (Table 1).

The most common types of grinding equipment were knife and rotor mills. The most widely used knife type was the Retsch GM 200 model, whereas the Retsch ZM 200 model was the most frequent rotor type. For the Retsch ZM series, different sizes of sieves were reported (from 0.5 to 2.0 mm). It was therefore decided to use these two main models.

Several grinding parameters were tested to define the optimal grinding conditions. The Retsch ZM 200 was tested with sieves of 0.5, 1.0, 2.0 and 4.0 mm operating at 14,000 rpm. The Retsch GM 200 was tested at various speeds (5000 and 10000 rpm) and durations (5, 10, 20 and 30 s).

Material

A representative feed had to be selected based on (1) a regular composition that excluded ingredients possibly interacting with the identification of bones; (2) being commercially available; and (3) having a sedi-

Table 1. NRLs' grinding equipment survey.

Type	Model	#
Knife mill	Grindomix (Retsch GM 200)	4
	Blender LB20E	1
	Ika M20	1
	Barbender	1
	Vertec	1
	Coffee mill	1
Rotor mill	Retsch ZM 100	1
	Retsch ZM 200	5
Hammer mill	Retsch SK 1	1
Other	Mortar	1

ment percentage close to a global 1.7% value for feedingstuffs (except fishfeeds) determined as per the official method (calculated on the results of about 350 feeds from the collection of the EURL-AP).

A commercial compound feed for rabbit matching these criteria was used for all experiments in this study. It was composed of wheat bran, pelleted alfalfa, sunflower cake, beet pulp, wheat, molasses, rapeseed cake, palm cake, soybean extraction cake, barley, calcium carbonate, mono-calcium phosphate and salt. Tetrachloroethylene sedimentation delivered a sediment content of about 1.6%. Light microscopic and PCR analyses confirmed this compound feed as negative for animal material (PCR targets used: ruminant, bovine, ovine, porcine, poultry, chicken–turkey and fish).

A PAP from commercial origin with a bone content of approximately 35% was selected. Light microscopy confirmed that the PAP only contained terrestrial animal material and PCR analyses indicated it only contained porcine and avian material.

Sample preparation

Preliminary study

For the preliminary study on the grinding parameters, the compound feed was used unadulterated (for the weight percentage estimation of the sieved fractions) as well as adulterated (for the weight percentage estimation of the sieved fractions of the sediment and the number of bones from the whole sediment).

The adulteration of the compound feed with the PAP at 0.01% and 0.05% w/w was performed by serial dilutions. In order to achieve a better homogeneity, all compound feed were coarsely ground at 4 mm by ZM 200 before initial addition of the PAP.

Collaborative study

Samples for the collaborative studies consisted of the compound feed adulterated with the PAP at 0.01% and 0.05% w/w. These levels of PAP concentration were chosen as they are both below the imposed 0.1% w/w that methods must be able to detect. As for the preliminary study, serial dilutions were used, after grinding of the compound feed at 4 mm by ZM 200, for achieving the adulteration level.

Study organisation

For each level of adulteration, 0.01% and 0.05%, three grinding treatments were applied: (1) 2 mm; (2) 0.5 mm with a ZM 200; and (3) at 10000 rpm for 10 s with a GM 200. Thus, six samples were produced. Each of the six samples was prepared in duplicate with one to be analysed unstained and the other after staining by Alizarin Red.

Each sample set sent to the participants consisted of 12 samples. Samples were labelled from 1 to 6 for Alizarin Red stained microscopic analyses and from 7 to 12 for unstained observations without disclosure of adulteration levels to the participants. The amount of each sample was 35 g, which allowed for three imposed repetitions of 10 g each. A total of 36 microscopic analyses per participant were required.

The required number of participants was fixed to a minimum of eight. Fourteen NRLs were invited to participate and 10 accepted and returned their results.

A write-protected file containing detailed instructions and a report form was sent to the participants. Among fixed instructions, sieving of the sediment was forbidden; each microscopic analysis had to be performed on three slides of the sediment prepared according EURL-AP standard operating procedure (SOP) (EURL-AP 2013); only bone fragments identified without any ambiguities were to be recorded.

Records that had to be encoded for each repetition were the total number of bone fragments from the three slides, the weight of the obtained sediment (stained or unstained) and the weight of remaining sediment after the slide preparation.

Data treatment and statistics

The normality of distribution of data was tested for each sample type by one-sample Kolmogorov–Smirnov tests as recommended by Sokal and Rohlf (1995). Repeatability and reproducibility were estimated according to ISO:13528. Data from the experiment were factorially analysed by a one-way general linear model analysis of variance (ANOVA) using SAS (v. 9.2) with the combination of the two factors ‘staining–grinding’ as the main factor (six levels). Due the lack of normality and inequality of variances, data were square root transformed. The

staining–grinding means were separated at the 5% significance level by the Newman–Keuls test.

Results and discussion

Preliminary study

All tests were performed on 10 g of the same compound feed matrix. The ground material was sieved through a series of sieves with different square mesh sizes (500, 250, 125, 75 and 50 µm). The weights of each sieved size fractions were collected and expressed as percentages (Table 2).

Results showed that regardless of the grinding process used, a large percentage (of weight) of coarse fragments (≥ 500 µm) remained. Regulation EC/152/2009 (EU 2009) imposes the use of a 250-µm sieve if fewer than 95% of the particles are smaller than 500 µm. To meet this condition, the only option was to grind the feed with a ZM 200 equipped with a 0.5-mm sieve grid. When using a GM 200, there is also a decrease in the percentage of that coarse fraction as a function of the increase of grinding time when the speed is fixed at 10000 rpm. This is not observed at 5000 rpm where the different durations used did not impact noticeably on any of the fractions. A higher speed of blade rotation ends up in more fragmentation according to an increase of grinding time. However, at this speed (10000 rpm) the lowest recovery rates were also observed (90% and 87%). The explanation for this is linked to the generation of a very dusty fraction that is lost as it sticks to the edge of the blade axis and the wall of the bowl.

The influence of the grinding parameters on the mass of stained sediment, obtained at targeted PAP adulteration levels of 0.01% and 0.05% w/w, was then investigated. Not all combinations of grinding parameters were used. Only five were selected: ZM 200 (with sieve grid of 2, 1 and 0.5 mm) and GM 200 with grinding duration of 10 s (at 10000 and 5000 rpm). For each combination a sample of 35 g was ground. A 10-g test portion of the ground material was then sedimented according to Regulation EC/152/2009 (EU 2009). Each sediment was stained by Alizarin Red and sieved with the sieves series previously mentioned. Sediment weights of each sieved size fraction were collected and expressed as percentages (Table 3).

Bone particles greater than 250 µm are less appropriate for proper slide preparation compared with smaller size particles. Furthermore, particles greater than this size are quite opaque to light and only the margins of the particles, which are more transparent, are useful for detecting morphological features allowing their identification as bones (e.g., lacunae). On the contrary, when particles are too small, less than 50 µm, they often lack these morphological markers. Therefore, it was considered that a particle size of less than 250 µm and greater or equal to 75 µm was probably the most interesting fraction for microscopic investigations. Table 3 shows that this size range represents roughly about 50% of the mass of stained sediment with the exception of the GM 200 grinding at 5000 rpm where this range is limited to 33–42% only. In the adulterated compound feed, the mean stained sediment content was 0.102 g. According to the EURL-AP SOP for slide preparation, this amount should theoretically be enough to prepare 10 slides based on 10 mg per slide as per SOP. However, as explained above, not

Table 2. Weight percentage of compound feed-sieved fractions.

			Weight percentage of sieved fractions (%)						
Grinder type	Sieve size or speed	Grinding duration (s)	≥ 500 µm	≥ 250 µm	≥ 125 µm	≥ 75 µm	≥ 50 µm	< 50 µm	Recovery rate (%)
ZM 200	4 mm		38.9	31.4	15.8	9.2	0.8	1.7	98
	2 mm		17.1	33.5	22.6	14.3	0.7	3.2	91
	1 mm		10.9	32.7	28.6	19.7	1.2	2.0	95
	0.5 mm		2.0	26.8	31.0	30.5	0.8	1.4	92
GM 200	10000 rpm	5	32.5	31.9	17.7	6.5	0.8	1.0	90
		10	24.0	34.0	21.0	8.8	1.5	1.6	91
		20	18.4	34.5	22.3	9.7	1.0	1.3	87
		30	15.3	33.1	25.8	14.0	1.9	2.3	92
	5000 rpm	5	38.7	30.1	14.8	7.1	0.9	0.4	92
		10	39.2	30.9	14.7	8.7	0.5	0.4	94
		20	39.3	30.2	14.5	7.9	0.8	0.4	93
		30	38.1	36.0	9.9	8.4	0.3	0.3	93

Note: Shaded cells are the grinding conditions retained for all other tests.

Table 3. Weight percentage stained sediment-sieved fractions.

Adulteration level (%)	Grinder type	Sieve size or speed	Weight percentage of stained sediment-sieved fractions (%) ≥ 500 µm	≥ 250 µm	≥ 125 µm	≥ 75 µm	≥ 50 µm	< 50 µm	Sediment [250 µm; 75 µm] (%)
0.01	ZM 200	2 mm	4.06	22.48	**38.24**	13.63	9.62	2.67	52
		1 mm	2.64	15.97	**38.30**	14.46	14.05	4.80	53
		0.5 mm	1.57	9.07	**35.07**	19.09	16.69	7.98	54
	GM 200 (10 s)	10000 rpm	5.76	31.26	**36.83**	10.41	5.67	3.49	47
		5000 rpm	9.87	**33.66**	33.39	8.87	5.30	2.60	42
0.05	ZM 200	2 mm	3.76	21.11	**36.68**	12.66	12.33	7.11	49
		1 mm	5.18	14.16	**33.19**	15.53	13.53	9.41	49
		0.5 mm	7.35	7.24	**29.80**	16.23	16.89	13.85	46
	GM 200 (10 s)	10000 rpm	8.82	27.49	**34.52**	10.32	8.44	4.13	45
		5000 rpm	23.68	**33.19**	25.88	6.79	4.41	2.14	33

Note: Figures shown in bold represent the highest values of mass fractions (%) per grinding condition.

all sieved mass fractions have the same value for microscopic purpose. Therefore, investigations on the value of each fraction of the stained sediment for the detection of bones were undertaken for each grinding process. The whole sediment was entirely observed by light microscopy and all bone fragments identified were recorded. These results are summarised in Table 4.

Interestingly, the coarsest fractions from the rotor mill treatments hardly contained any bones as compared with the knife mill where 5–8% of bone fragments were found. Regardless of the grinding treatment and the adulteration level used, the largest percentages of bone fragments were found in fractions less than 250 µm and greater or equal to 75 µm. These fractions accounted for 73% of the total bone fragments found and identified. The total number of bone fragments ranged from 12 to 32 for the 0.01% PAP adulterated feed and from 66 to 392 for the 0.05% PAP-adulterated feed. The largest total numbers of bone fragments were observed with rotor mill grinding at 0.5 mm. However, variations among these numbers of bones were found and, putting aside the strict effect of grinding, the following explanations may account for this. Firstly, the initial number of actual bones is unknown as the bone content may slightly vary between the initial amount of PAP material used for the adulteration, or initial spiking, especially when at low level of adulteration, as has been the case in this study. Secondly, sediments were stained and this process may result in some loss of bone particles. Thirdly, the homogeneity of the distribution of bone fragments is an unknown parameter. Finally, on the fine fractions, below 50 µm, some bones are no longer recognised due to the loss of morphological features linked to the size reduction.

From this preliminary study, it was decided to prepare samples delivering, respectively, high, medium and low total numbers of bone fragments: ZM 200 at 0.5 and 2 mm sieves, and GM 200 at 10000 rpm for 10 s.

Collaborative study

On the hypothesis that the homogeneity of the sediment is satisfied, then logically the more material from the sediment that is used for slide preparation, the more bone fragments will be found. Provided

Table 4. Relative distribution (%) of identified bone fragments in stained sediment-sieved fractions.

Adulteration level (%)	Grinder type	Sieve size or speed	Bone fragment percentage in sieving fractions (%) ≥ 500 µm	≥ 250 µm	≥ 125 µm	≥ 75 µm	≥ 50 µm	< 50 µm	Useful bone percentage [250 µm; 75 µm] (%)	
0.01	ZM 200	2 mm	0	23 (6)	46 (12)	19 (5)	12 (3)	0	65	Mean = 73
		1 mm	0	22 (4)	56 (10)	11 (2)	11 (2)	0	67	
		0.5 mm	0	9 (3)	47 (15)	41 (13)	3 (1)	0	88	
	GM 200	10000 rpm	8 (1)	17 (2)	25 (3)	50 (6)	0	0	75	
		5000 rpm	5 (1)	5 (1)	23 (5)	45 (10)	14 (3)	9 (2)	68	
0.05	ZM 200	2 mm	1 (2)	6 (10)	29 (51)	45 (80)	17 (30)	2 (3)	74	Mean = 73
		1 mm	1 (2)	6 (13)	29 (59)	47 (95)	13 (27)	3 (7)	76	
		0.5 mm	0	2 (9)	31 (120)	47 (183)	17 (66)	4 (14)	77	
	GM 200	10000 rpm	5 (6)	6 (7)	32 (35)	39 (43)	13 (14)	5 (6)	70	
		5000 rpm	5 (3)	3 (2)	20 (13)	45 (30)	23 (15)	5 (3)	65	

Note: Numbers in parentheses are the actual number of bone fragments. Shaded columns represent the most valuable size fractions for light microscopic observations.

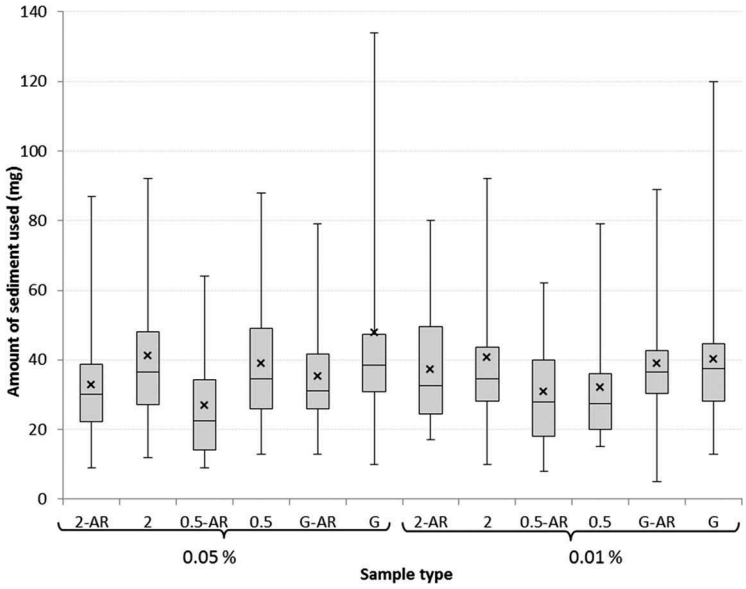

Figure 1. Box plots of the amount of sediment used for preparing three slides. 2 = ZM 200 at 2 mm; 0.5 = ZM 200 at 0.5 mm; G = GM 200; AR = Alizarin Red staining.

this assumption is respected, comparisons of the numbers of bone fragment can only be made if the amount of sediment used is the same. From the raw results it appears that it was not the case (Figure 1).

According to the SOP, it is recommended that one uses about 10 mg of sediment per slide. This recommendation was followed by a majority of participants: for the three slides the overall mean amount of sediment used through the 360 performed analyses was of 36.9 ± 2.0 mg. However, two participants systematically used much more than this prescribed amount, with means of 64 and 69 mg respectively. These participants also had all the maxima observed for each sample type (Figure 1). In order to avoid biases, it was thus decided to report all bone counts on a same standard amount of 30 mg. Transformation of the data was achieved by simply dividing the number of bone fragments found by the amount of sediment used to obtain an expected number of bones per mg, which was then multiplied by 30 to obtain the final converted number of bone fragments. The normality of the distribution of the number of bone fragments was then controlled on each sample type. Not all were normally distributed, but only nine of the 12. Due to this fact, robust statistics were used to study repeatability and reproducibility (Table 5).

Table 5. Repeatability and reproducibility of the number of bone fragments detected.

			Average (± 2 SD)	s_r	RSD_r (%)	s_R	RSD_R (%)
0.05%	2	AR	62.97 (± 21.99)	9.65	15	34.77	55
		Unstained	30.15 (± 13.17)	7.47	25	20.82	69
	0.5	AR	137.88 (± 46.62)	20.29	15	73.71	53
		Unstained	43.76 (± 22.38)	10.10	23	35.38	81
	G	AR	35.37 (± 15.11)	9.68	27	23.89	68
		Unstained	15.47 (± 7.02)	2.66	17	11.09	72
0.01%	2	AR	8.81 (± 3.32)	2.69	30	5.26	60
		Unstained	4.48 (± 2.88)	1.44	32	4.56	102
	0.5	AR	18.25 (± 6.94)	6.93	38	10.97	60
		Unstained	13.33 (± 7.30)	4.09	31	11.54	87
	G	AR	6.02 (± 2.89)	2.12	35	4.56	76
		Unstained	2.53 (± 1.68)	0.48	19	2.66	105

Notes: All data are expressed in numbers of bone fragments.
2 = ZM 200 at 2 mm; 0.5 = ZM 200 at 0.5 mm; G = GM 200; AR = Alizarin Red staining; average = robust mean of all submitted results; SD = standard deviation of the average, calculated from the reproducibility SD divided by the square root of the number of laboratories; s_r = repeatability SD (within-laboratory variability); RSD_r = relative repeatability SD; s_R = reproducibility SD (within plus between laboratory variability); RSD_R = relative reproducibility SD.

The repeatability based on the three repetitions on each sample type and expressed by the relative repeatability standard deviation (RSD_r) ranged from 15% to 27% for the 0.05% adulteration levels and from 19% to 38% for the 0.01% adulteration levels. These values for the repeatability are acceptable. There was a trend for a better repeatability for the higher level of PAP adulteration.

The reproducibility expressed by the relative reproducibility standard deviation (RSD_R) ranged from 53% to 81% for the 0.05% adulteration levels

and from 60% to 105% for the 0.01% adulteration levels. RSD_R values were always higher for unstained sediments than for Alizarin Red-stained ones, thus reflecting a slightly improved reproducibility when staining is performed. However, considering the values, this improvement of reproducibility is not substantial. The lower RSD_R values are partly linked to the systematically observed higher number of bones from the three slides of stained versus unstained sediment, as reflected by the averages or robust means.

Analysis of the distribution of the number of bones (Figure 2) shows a generalised right skewness of the data, which might indicate a possible non-normality of the data. It held true whatever the grinding conditions, staining or absence of staining and levels of adulteration. For each treatment few occurrence of high bone numbers were reported. The absence of normality was confirmed for 25% of the cases as mentioned. Observed means for the stained samples were, with the sole exception of 0.01% adulterated sample ground with the GM 200, always greater when compared with unstained samples. Sometimes this mean number of bones was up to 3.25 times greater as for the 0.05% adulterated sample ground at 0.5 mm. However, the real significance of these differences of means can only be evaluated under the assumption of equality of variances; this homoskedasticity failed to be demonstrated. Therefore, ordinary ANOVA could not be used and non-parametric tests, after square root transformation to normalise the data, had to be performed.

Comparison of square root-transformed data (Figure 3) by Newman–Keuls tests showed that at 0.05% of PAP adulteration the only significantly (at $p = 0.05$) differing means where those observed on the samples ground at 0.5 mm and stained (highest mean) and the samples ground with the GM 200 unstained (lowest mean). At 0.01% of PAP adulteration, only the sample ground at 0.5 mm and stained (highest mean) was significantly discriminated from any other treatment combination. All other treatment combinations were not affecting the mean numbers of bones. This confirms assumptions that could already be made from the averages or robust means presented in Table 5.

Considering from the preliminary study that the ZM 200 grinding with 0.5 mm mesh sieve was the only grinding treatment delivering > 95% of particle size inferior to 500 µm, these findings are not surprising as from such a grinding condition there is a

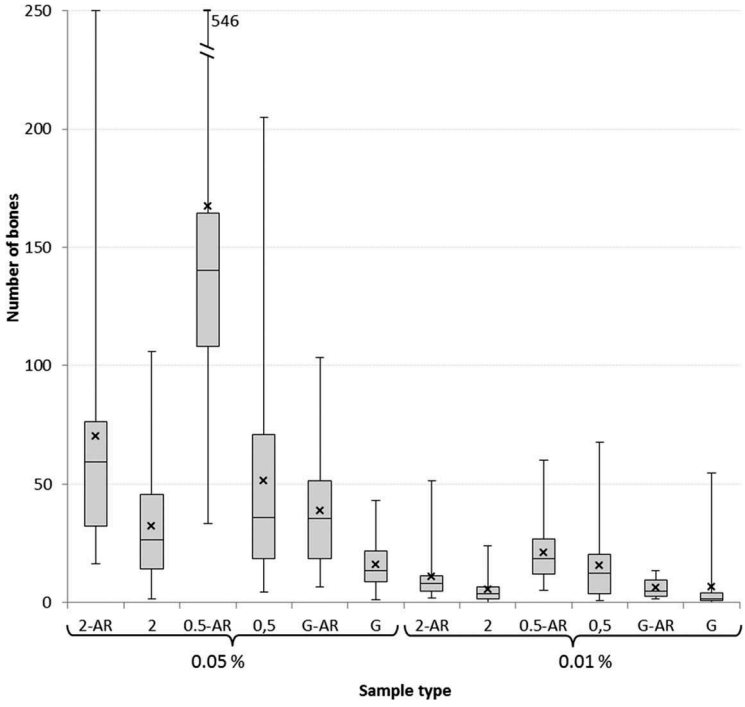

Figure 2. Box plots of the number of bone fragments detected in 30 mg. 2 = ZM 200 at 2 mm; 0.5 = ZM 200 at 0.5 mm; G = GM 200; AR = Alizarin Red staining.

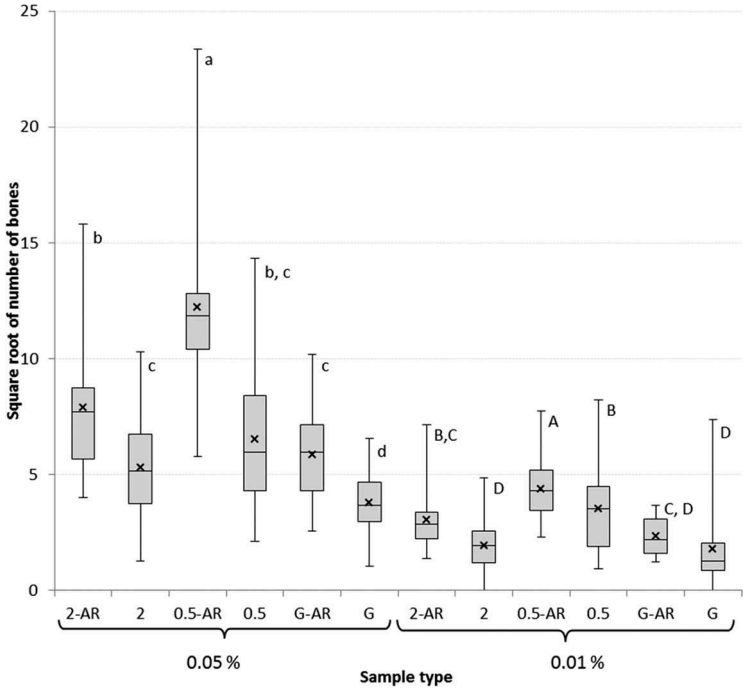

Figure 3. Box plots of the number of bone fragments detected in 30 mg after square root transformation. Sample types with the same letter do not differ from each other (at $p = 0.05$). Lower case letters are used for 0.05% samples and upper case for 0.01% samples.

fragmentation of the bones into smaller spicules. However, more questionable is the fact that at 0.5 mm grinding only the Alizarin Red-stained sediment was significantly different from the other combinations of grinding–staining at both percentages of adulteration. ANOVA demonstrated that only grinding had a significant impact on the mean number of bones. Staining only resulted in trends to a greater number of bones and no significant impact of the interaction of grinding–staining could be detected.

When these results are considered with regard to the requirements of the official method, as described in EU Regulation EC/152/2009 and related SOPs, one can only conclude that the effect of grinding is on the whole minor or at least insignificant. As a reminder, the legislation authorises only a sample to be reported as positive if more than five particles of a given nature (i.e., terrestrial or fish) are found on average through the observation protocol. In line with EFSA risk assessment as regards transmissible spongiform encephalopathies (TSE) (EFSA 2011), the method must be able to detect PAPs in feed at a minimum level of 0.1% w/w. This study was based on the minimal conditions of observations, three slides from the sediment, as imposed by the current legislation.

Under these conditions, from the 180 determinations based on 30 mg of sediment made at 0.05% of adulteration, 173 of them would directly and officially have to be reported as truly positive. Only seven determinations delivered fewer than five bone particles. However, since the observation protocol imposes repetitions in such situation, from those seven cases only four would have been reported as truly positive and only three cases would have been reported as below the LOD of the method. These three remaining cases originated from a single participant on the sample ground with the GM 200 and on unstained sediment. Finally, no situation in which bones failed to be detected, i.e., a false-negative result, was recorded.

For the 180 determinations made under the same conditions at 0.01% of adulteration, the situation is different: 66 determinations delivered fewer than five bone particles and 10 failed to detect any bones. However, due to mandatory repetitions, seven of these 66 cases would have been turned up as truly positive. For the 10 cases where no detection of bone was reported, five came from the sample ground at 2 mm and five from the sample ground with the GM 200, each time on unstained sediments. All 10 false-negative results originated from two

participants only. For one of these two participants, collecting six false-negative results, it was noted that the amount of sediment used to prepare the series of three slides was insufficient: a mean of 16 mg per series instead of the endorsed 30 mg. This could have explained the false-negative results, although in this study no correlations could be observed between the amount of sediment used and the number of bones reported – regardless of the PAP adulteration level, the type of grinding, and the staining or absence of staining (data not shown). This absence of correlation can be explained by the low adulteration levels, both below the commonly referred LOD of 0.1%. Low concentrations of analytes are known to generate a wider variability or dispersion of results, as also demonstrated in the present repeatability–reproducibility data.

Conclusions

The present study showed that grinding had a limited effect on the number of bones that can be counted from slide preparations made of sediment from PAP-containing feed. The anticipated effect of bone fragmentation due to grinding, as shown by the Irish model experiments with bone spicules as well as data on the total bone numbers from the present preliminary study, could not be replicated under the conditions of the collaborative study. This, however, does not question the Irish model study. It only demonstrates that real-world grinding conditions of PAPs in compound feed may deviate from such a model – based on an exact number of bone spicules added to simple feed matrices. Applied to real PAPs, the only significant difference found was when rotor mill grinding at 0.5 mm was applied and Alizarin Red staining performed on the sediment prior to slide mounting. None of the other grinding–staining conditions could significantly impact the results. However, one needs to consider that the feed matrices (hardness differences notably) may also have an impact on the grinding. This aspect could not be addressed in this work since it was based on a single-compound feed matrix. Results from the collaborative study showed good repeatabilities for the number of bones. Reproducibility for the number of bones was poor as might be expected from the low levels of adulteration. It also did not reveal any relationship between the amount of sediment used and the number of bones, or any convincing influence of Alizarin Red staining on the number of bones detected.

With reference to the legislation imposed for light microscopic detection of PAPs in feed, the grinding and sieving conditions do not need further detailed descriptions or recommendations. The mandatory use of a 250-µm sieve to separate a fine fraction from a coarse one has to be maintained. Effectively, the only grinding condition of the study that enabled more than 95% of particles to be below 500 µm was by rotor mill grinding at 0.5 mm. Under this condition, 250-µm sieving is not required, while for all other grinding conditions it is. This study also showed that on average more than 70% of all recognisable bones were found in the fraction between 250 and 75 µm whatever the grinding process. The amount of material referred into the SOP to prepare slides, circa 10 mg per slide, is enough to conclude on the presence of bones in feed. Considering that operational schemes impose also to prepare slides from other fractions (flotate of raw material), thus enabling identifying more particles from terrestrial or fish origins to sum up to the bone detection, there is no need to increase the quantity of sediment provided the slide mounting SOP is fully followed – including the indicated amount of material. However, the study highlighted that using less material than requested leads to erroneous negative results. Fixing an imposed minimal amount of material would prevent analysts from such situations. Furthermore, at 0.05% of PAP adulteration of feed, which is half the requested legal LOD of the method with respect to the TSE risk assessment, in 96% of cases the analyses could have been limited to a single determination since more than five bone particles were already observed (at 0.01% a single determination represented already 56% of true-positive results). Regarding the use of the Alizarin Red staining, although at first glance it seems to double the number of bones compared with unstained conditions, its effect is, however, only a trend since it is statistically not significant.

With regards to other possible method improvements, this study being limited to the use of only one PAP added to a single-feed matrix does not allow any further recommendation or opinion.

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