Community Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Quality of Agricultural Products Department
Chaussée de Namur 24, 5030 GEMBLOUX, Belgium

☎32 (0) 81 62 03 74 32 (0) 81 62 03 88
e-mail: secretary@crl.cra.wallonie.be Internet : http://crl.cra.wallonie.be

CRL-AP Interlaboratory Study 2007
Final report

Authors:
P. Veys* and V. Baeten*

Contributors:
G. Berben*, A. Boix† and C. von Holst†

June 2008

* CRL-AP, CRA-W, Gembloux, Belgium
† DG – Joint Research Centre – IRMM, Geel, Belgium

ISBN 978-2-87286-067-8
Dépôt légal D/2009/1463/3

Edité par :

Centre wallon de Recherches agronomiques
Service Communication
Rue de Liroux, 9
5030 Gembloux (Belgique)

Summary

On behalf of DG Sanco, the Community Reference Laboratory for animal proteins in feedingstuffs (CRL-AP) organized in 2007 the present interlaboratory study for all National Reference Laboratories (NRLs) of the European Union. The goal of the study was to evaluate and to validate a revised protocol for the quantification of processed animal proteins in feed based on that proposed by the reference method as stated in EC 126/2003. This enhanced protocol included a determination of the *d* factor based on a grid counting process and the use of a standard calculation tool for the final estimation of adulteration by animal proteins.

The initial number of participants was 26 but only 22 result sets were considered. Each participant received 10 blind samples of materials adulterated by fish meal at different levels of concentration. Instructions were to apply strictly the revised protocol for quantification. No qualitative analyses were asked to the participants.

The study showed that both grid counting for the calculation of *d* and the calculation tool for the final percentage estimation of fish meal are appropriate for better standardization of measurements. When compared to the CRL-AP Interlaboratory Study 2006 improvement of the global reproducibility was noted but the study failed at increasing a nonetheless acceptable repeatability. Furthermore a generalized overestimation of the calculated percentages was observed. Therefore the protocol could not be validated. Different possible causes were identified for explaining this overestimating trend but all rely eventually on human skills such as the ability to identify correctly the origin of particles. Actually the study established that the correct evaluation of *d* was demonstrated to be critical as it entirely rely on the microscopists' ability to characterise the origin of a particle.

Once again this study highlighted the need of experience: from the 5 participants that presented poor results, 3 of them did not participate to the former interlaboratory study on quantification.

Keywords :

Meat and bone meals – Processed animal proteins – Microscopy – Quantification – EC 126/2003 directive – protocol improvement

1. Foreword and aim of the study

Community Reference Laboratories (CRL) were created in order to ensure a high level of quality and an uniformity of the results provided by European control laboratories. On 29 April 2004, the European Parliament and the Council have adopted the Regulation (EC) No 882/2004, improving the effectiveness of the official food and feed controls while redefining the obligations of the relevant authorities and their obligations in the organization of these controls.

On 23 May 2006, the Commission Regulation (EC) No 776/2006, has nominated the Walloon Agricultural Research Centre as Community Reference Laboratory for animal proteins in feedingstuffs (CRL-AP, http://crl.cra.wallonie.be) for the 2006-2011 period. The new Community Reference Laboratory has to develop the following priority axes:

(i) To provide National Reference Laboratories (NRLs) with detailed analytical methods, including reference methods for the network of Member State NRLs;

(ii) To coordinate application by NRLs of the methods by organizing interlaboratory studies;

(iii) To develop new analytical methods for the detection of animal proteins in feedingstuffs (classical microscopy, near infrared microscopy, polymerase chain reaction, immunology …);

(iv) To conduct training courses for the benefit of NRL staffs from Member States and future Member States;

(v) To provide scientific and technical assistance to the European Commission, especially in cases where Member States contest the results of analysis.

In this framework in 2006 the CRL-AP organized an interlaboratory study which demonstrated some shortages of the quantification method of animal constituents in feed from the EC 126/2003 directive [1]. Based on discussions with the NRL network during the first annual CRL-AP Workshop, it was decided that the CRL-AP would undertake research on possible enhancements of the quantification. The obtained results from a preliminary internal study lead to a revision of the quantification method.

Major enhancements are based on:

- the application of grid counting as base for the estimation of *d* factor and
- the use of a standard calculation tool.

The objective of the present CRL-AP Interlaboratory Study 2007 is to evaluate this revised protocol for the quantification of processed animal proteins in feed based on that proposed by the reference method as stated in EC 126/2003.

Results of this study were presented and discussed with the participants during the 2nd CRL-AP Workshop organised at Gembloux, Belgium, from 15 April to 17 April 2008. The present final report, based on a working document diffused to all NRL, includes some enhancements that were made during the discussions of the workshop.

2. Introduction

The study was officially announced to each participant (cf. list in Annex 1) on the 15 October 2007. Aside the general outline of the interlaboratory study and classical recommendation such as their responsibility for sub-sample homogeneity, the announcement also informed the participants on the type of grid reticle they had to order for the purpose of the study.

The detailed text instructions (Annex 2) and a dedicated Excel calculation tool (Annex 3) were posted on the CRL-AP intranet on the 19 October. The content of the information posted was the following:

- Description of the general principle of the method (no qualitative analysis, only quantification),
- The list of additional equipment needed, such as the eyepiece reticle counting pattern NG14,
- The detailed steps for obtaining the sediment which had to be stained by Alizarin red and weighed before and after staining,
- The detailed slide mounting and preparation on the two sieved fractions from the stained sediment as well as the number of slides that had to be prepared,
- The principles and the instructions for the correct grid counting process (including general theoretical notions, pitfalls to avoid, number of fields to observe, randomized field selection),
- The method and formula used for the calculation of the estimation of the portion of terrestrial and fish bones from the stained sediment (respectively the *c* and *d* factors),
- The way those factors are taken into account in the final formula for the calculation of the value of constituents of animal origin,
- Instructions on the use of the Excel calculation tool provided to the participants of the study.

A PowerPoint slideshow was also provided on that same date in order to give an illustrated version of the text instructions and all requirements for a correct grid counting as well as the use of the calculation tool.

A two month training period was planned allowing time for participants to become confident with the grid counting process. Due to lack of commitment from the grid provider and the unacceptable long delivery delays that were reported to the organizer, the deadline for returning the results to the CRL-AP was postponed in agreement with the Commission to the 21 January 2008 (instead of the 20 December 2007 as initially planned).

Samples however were sent on initial date to the participants, i.e. on the 30 November 2007. Thus each participating lab received a set of 10 blind compound feeds fortified with fish meal at various levels of concentrations as stated in the announcement.

Participants were asked to encode their results by way of an Excel report form -downloadable from the CRL-AP intranet (Annex 4). Participants were asked to carefully read the instructions on how to fill in the result form and to testify they did it prior to encoding their results. No other support for communicating the results was accepted. A summarized results sheet was automatically generated without the need for the participant of re-encoding the data. Participants were asked to sign the summarized results sheet and to send it by fax to the CRL-AP. Results were taken into consideration only when both the Excel file and the fax were received.

According to the difficulty encountered by some participants for obtaining the eyepiece reticle, the organisers accepted results from few participants somewhat later than on the due date. In each of those few cases the organisers were informed by the participants themselves prior to the 21 January 2008. Nevertheless results were no more accepted beyond the 7 February 2008.

Thus on the 26 participating laboratories, only results from 22 labs were accepted.

- Two labs were considered as excused (labs number 15 and 20).
- Lab 19 was excused because of an ICT problem (the Excel report form could not be transmitted but only the fax).
- Lab 7 did not report its results. The Commission was informed on this situation.

3. Material and methods

3.1. Material

3.1.1. Description of the samples

Five different samples containing typical compound feed and fish meal at different concentration levels have been prepared as shown in table 1.

The composition was established taking into account the following features for the quantitative method assessment:

- Target concentrations of fish meal were selected at 4 different levels: 0.15%, 0.40%, 0.70% and 1.00% in order to evaluate the potential of the method assuming that a tolerance level of 1% could be introduced. The chosen percentage of adulteration differs from the classical 0.10%, 0.25%, 0.5% and 1% series in order to avoid any participant's anticipation on the adulteration level.
- The choice of two different compound feeds in order to obtain a matrix effect on the quantification.

Each participating lab received about 55g of 10 blind samples to which a unique random number was assigned. Details of the samples are indicated in table 1.

Table 1: Composition of the blind samples set used in the CRL-AP Interlaboratory Study 2007.

Sample	Material	# replicates
A	0.15% Fish	2
B	0.4% Fish	2
C	0.7% Fish	2
D	1% Fish	2
E	1% Fish in matrix 06	2
Total		**10**

3.1.2. Materials used in the preparation of the samples

The **base of the test material** for samples A, B, C, D was a classical compound feed produced under strict controlled conditions in a pilot plant. The matrix was composed of wheat, corn, soya bean meal, fat of plant origin, limestone, salts, minerals and vitamins. Sediment content of the compound feed was about 1.46% (STD 0.08%) after staining with Alizarin red. Prior to use, the material was tested by classical microscopy and Polymerase Chain Reaction (PCR) in order to confirm the absence of any interfering substances.

Another classical compound feed for sample E was the base feed used in CRL-AP ILS 2006 study. This feed, also produced from a pilot plant, contains corn, soya beans and hulls, rapeseed, beet, straw, molasses, sunflower, palmkernel, coconut, citruspulp, wheat, vegetable fat and various feed additives such as limestone, salts, minerals and vitamins. Purity and quality of this material has been studied during the CRL-AP ILS 2006 study [2].

The **fish meal** used in the study was the Fish I meal used in CRL-AP ILS 2006 study [2]. The fishbone content was of about 12%. Purity of the fish meals was investigated in the CRL-AP ILS 2006 study [2].

3.1.3. Description of the mixing procedures

Prior to their use in mixes, the feed matrixes and the fish meal were sieved in order to keep only particles from the 0 – 1000 µm fractions.

The **stepwise dilution procedure** developed by CRA-W and JRC-IRMM was used to produce the materials A, B, C and D fortified with fish meal. This procedure has been successfully used in five former European interlaboratory studies (DG-Sanco 2003 [3], DG-Sanco 2004 [4], STRATFEED [5] studies, CRL-AP ILS 2006 [2], CRL-AP PT 2007 [6]).

The **spiking procedure**, as described in CRL-AP ILS 2006 [2] was used for the preparation of samples of material E.

3.2. Quantitative analysis

The objective for the present interlaboratory study was to assess the revised quantification method from EC 126/2003 directive based on the standard use of grid counting and a provided calculation tool. The ultimate goal is thus that of a quantitative method validation. Therefore labs were explicitly asked to strictly follow the protocol they were given (Annex 2).

Each participant had to realise 10 quantifications on each two replicates of each five samples.

Calculations had to be made by way of the Excel calculation tool by input of the following data according to the instructions:

- W, weight of the sample used for obtaining the sediment (10 g at 0.01g)
- S2, weight of the stained sediment (at 0.001g)
- The number of counts for each type of particles (other and fish) observed through the 20 randomly chosen fields (5 fields per slide on 4 slides), from which the value of *d* factor was automatically calculated

The results, computed by the calculation tool with an assigned default value for *f* factor of 0.10, were expressed in terms of estimated value (in % at 0,001%) of constituents of fish origin, in compliance to the EC 126/2003 directive.

As for a previous study on the quantification realised by the CRL-AP [2] the organisers wanted to consider all results from the analysis and thus chose for robust statistics analyses [7]. The advantages of those statistics in comparison to the traditional approach have already been presented in former studies [2].

The estimates of the mean values and the precision of the data expressed in terms of within-laboratory variation (*repeatability* standard deviation) and between-laboratory variation (*reproducibility* standard deviation) are obtained by analysis of variance (ANOVA) as specified in the IUPAC guideline for method validation [8]. Robust statistics have been applied to the estimation of the standard deviation and the average.

An indicative proficiency for each participant was assessed by *z*-score analyses. The global performance was estimated by pooling of *z*-scores and obtaining the *z*-score global mean or rescaled sum of scores (RSZ) [9] for each participant. The relative laboratory performance (RLP) [9] was also investigated in order to confirm which participant delivered repeated deviating or questionable results.

4. Results

Gross results forms from all participants are to be found in Annex 6.

4.1. Homogeneity study

The protocols used for preparing the different materials are known for delivering good homogeneities as proved by previous studies [2, 3, 4, 5, 6]. The homogeneity was checked on two materials the 0.4% Fish and 1% Fish. From each material, 5 samples of 10g have been sedimented. Permanent slides were prepared from the whole stained sediment and quantification has been performed according the protocol. From each slide 6 random fields were analysed. This has been repeated 3 times and by 2 different operators. Table 2 gives the summary of those quantifications. The achieved results were considered as acceptable.

Table 2: Homogeneity study – Quantification results.

	0.4% Fish				1% Fish			
Sample	**nb slides * (# <250µ + # >250µ)**	**S2/W (%)**	**d (%)**	**Fish content (%)**	**nb slides * (# <250µ + # >250µ)**	**S2/W (%)**	**d (%)**	**Fish content (%)**
1	10 (6+4)	1.57	2.10	**0.33**	11 (6+5)	1.62	4.63	**0.75**
2	8 (4+4)	1.78	2.13	**0.38**	10 (5+5)	1.65	5.97	**0.99**
3	8 (4+4)	1.63	2.02	**0.33**	11 (7+4)	1.75	4.36	**0.76**
4	9 (5+4)	1.80	1.79	**0.32**	10 (5+5)	1.78	5.52	**0.98**
5	8 (4+4)	1.62	2.52	**0.41**	11 (6+5)	1.65	5.59	**0.92**
mean		1.68	2.11	**0.35**		1.69	5.21	**0.88**
STD		0.10	0.26	**0.04**		0.07	0.69	**0.12**

For each slide 36 random fields were observed (= 6 fields/slide x 2 operators x 3 repetitions).

**** from the whole sediment***

4.2. Quantification results

4.2.1. Preliminary remark

For reminder the main goal of this interlaboratory study was that of assessing the robustness of the revised quantification method of EC 126/2003 directive and not that of evaluating the participants as it would have been the case for a proficiency study. Therefore accordingly the preceding any casual ranking of participant in this document is purely informative.

The formula used by the participants through the calculation tool is simply adapted from that of EC 126/2003 directive to use for the calculation of the estimated value (in %) of constituents of fish origin is the following:

$$\% = \frac{S2 \times d}{W \times f} \times 100$$

Where *S2* is the weight of the dry sediment after Alizarin red staining (in g at 0.001g), *W* the weight of the sample material for the sedimentation (fixed to 10g at 0.01g), *d* is the correction factor for the estimated

portion of fish bones and scale fragments in the sediment (in %), *f* is the correction factor for the proportion of bones in the constituents of animal origin in the sample examined (fixed to 0.10 in the study).

From this formula and given the study conditions (imposed *W* and *f* values), it appears that the remaining possible sources of variation are limited to *S2* and *d* – the latter being automatically computed by data encoded in the calculation tool by using the following formula:

$$d = \frac{Fc}{Fc + Oc} = \frac{Fc}{\Sigma\ all\ counts}$$

Where *Fc* is the number of counts for fish particles, *Oc* the number of counts for particles of other nature determined during the grid counting process. In order terms, *d* relies entirely on the distinction skills of the microscopist.

Reported percentages were recorded at 0.001% without any rounding.

4.2.2. Time required for the quantification

Table 3: Total time spent on the whole set of 10 samples, expressed in days.

Lab ID	Time (d)	Lab ID	Time (d)
1	4.5	18	10
2	15	21	5
5	4	22	3
6	2	23	3
8	3	24	2
10	16	28	3.5
11	10	29	3
12	3	30	3
13	2	33	7
14	1.5	34	3
17	10	35	10

One of the issues this study wanted to point out was that of trying to reduce as far as possible the time spent on a set of 10 quantifications. This might seem casual but from the preceding test including quantitative analysis (CRL-AP ILS 2006) many NRLs claimed that the charge of work was too high at least for routine quantification. From the preliminary study carried out for the establishment of the protocol, the CRL-AP experienced by using the grid counting a gain of time. The work charge for the quantification was approximated to be reduced by two times. Therefore it was asked to the participants to report the total time used for the whole study on the exclusion of the reporting. The results are illustrated in table 3.

The range of time varies between 2 days and 16 days for realising the whole work on the 10 replicates. The mean value of 5.6 days means that 2 samples can be handled by day. From informal discussions with different lab microscopists this seems acceptable, knowing that quantification requires a lot of concentration efforts and that from internal CRL-AP experience a maximum a 3 quantifications a day is yet a real performance for a single operator. Some other labs nevertheless consider this protocol to take too much time especially when considering the staining steps.

4.2.3. Reported percentages of constituents of fish origin – or gross results

The estimated values of constituents of fish origin as reported by the 22 participants are shown in table 4 (next page).

Table 4: Quantitative results. The upper part table gives the results expressed in %. The two maximal values for a sample are indicated in bold red, whereas the two minimal values are in bold blue (this hold true for all tables in this document). The lower part table provides some basic statistics (Legend: n = nr of quantifications, STD = standard deviation, min = minimum value, max = maximum value)

	0.15 % Fish		0.4 % Fish		0.7 % Fish		1 % Fish		1 % Fish 2006	
Lab ID	I	II	I	II	I	II	I	II	I	II
1	0.261%	0.134%	0.651%	0.636%	1.417%	1.132%	1.142%	1.368%	1.197%	1.055%
2	0.626%	0.484%	0.968%	0.730%	1.282%	0.884%	1.447%	0.937%	2.056%	2.268%
5	0.358%	0.532%	0.716%	0.905%	1.308%	0.750%	2.060%	1.173%	1.212%	2.294%
6	0.684%	0.485%	0.522%	0.640%	0.617%	0.914%	0.855%	0.725%	1.432%	1.219%
8	0.421%	0.863%	1.285%	1.429%	1.607%	1.855%	1.966%	1.659%	1.538%	1.790%
10	**0.005%**	0.006%	**0.030%**	**0.006%**	**0.028%**	**0.026%**	**0.051%**	**0.034%**	**0.077%**	**0.052%**
11	1.281%	0.623%	1.331%	1.834%	1.668%	2.725%	1.355%	1.325%	1.936%	2.596%
12	0.037%	0.039%	0.140%	0.957%	0.950%	0.535%	0.694%	0.860%	1.075%	0.675%
13	**0.000%**	**0.000%**	0.168%	0.614%	0.061%	0.448%	0.767%	1.713%	1.281%	1.522%
14	**1.773%**	0.556%	1.531%	1.005%	1.842%	2.360%	2.846%	2.462%	1.582%	1.461%
17	0.166%	0.270%	0.499%	1.130%	0.587%	0.937%	0.961%	1.008%	1.425%	1.290%
18	1.502%	1.695%	2.656%	**3.308%**	**4.682%**	2.680%	**5.732%**	3.925%	2.704%	2.962%
21	**1.831%**	1.541%	2.788%	1.736%	**4.448%**	3.072%	5.216%	4.304%	**3.432%**	2.338%
22	0.708%	0.356%	2.303%	1.736%	2.165%	2.067%	3.223%	2.458%	1.383%	2.499%
23	0.610%	0.606%	0.311%	0.596%	0.937%	1.067%	1.476%	0.335%	0.376%	1.259%
24	0.455%	0.105%	0.338%	0.171%	0.056%	0.400%	0.970%	0.846%	0.779%	0.711%
28	0.642%	0.971%	0.881%	0.885%	1.223%	1.150%	1.698%	1.842%	0.942%	1.434%
29	1.468%	0.214%	0.463%	0.951%	0.772%	0.225%	0.243%	2.223%	0.878%	1.568%
30	0.650%	0.267%	0.647%	0.961%	0.736%	0.677%	1.357%	1.561%	1.181%	1.134%
33	0.676%	0.291%	1.005%	1.961%	2.074%	2.153%	3.169%	2.910%	2.501%	2.118%
34	1.077%	0.173%	1.094%	1.989%	2.213%	2.661%	**6.408%**	3.339%	0.954%	2.574%
35	0.551%	1.196%	1.365%	**4.118%**	2.759%	1.936%	3.604%	3.154%	1.310%	**3.626%**

n	44	44	44	44	44
mean	0.618%	1.136%	1.457%	1.986%	1.584%
STD	0.517%	0.878%	1.077%	1.479%	0.808%
min	0.000%	0.006%	0.026%	0.034%	0.052%
max	1.831%	4.118%	4.682%	6.408%	3.626%
median	0.542%	0.954%	1.187%	1.519%	1.429%

Basic statistics (lower part table 4) shows heterogeneity among results. The means of all samples, although increasing according to the attended values, indicate a general overestimation among results. This is also reflected by the fact that the medians are always lower than the means. The standard deviations which are almost equal to the means indicate a very high variability between the results.

Some labs systematically present maximal and minimal values:

- Lab 10 has almost all severe underestimation (9 on10)
- Labs 18 and 21 have repeated maxima (3 on 10)

Heterogeneity of results appears also when looking between two replicates values of one sample (e.g. lab 35 for the 0.4% Fish and 1% Fish 2006: one replicate is about 3 times the other, lab 34 for the 1% Fish: one replicate is about twice as high as the other).

At first sight the variability of results may both be linked to between-laboratory and within-laboratory variability's.

Nevertheless it seems that some labs present too many repeated abnormal values and might therefore be considered as potential outliers. Therefore robust statistics were applied in order to take the suspected aberrant values into consideration.

Table 5: Robust statistics results on the reported percentages of constituents of fish origin.

	Average	STD	s_r	RSD_r	s_R	RSD_R
0,15% Fish	0.570 (± 0.20)	0.10	0.303	53	0.480	84
0,4% Fish	1.029 (± 0.30)	0.15	0.434	42	0.716	70
0.7% Fish	1.374 (± 0.42)	0.21	0.357	26	1.000	73
1% Fish	1.829 (± 0.54)	0.27	0.499	27	1.282	70
1% Fish 2006	1.557 (± 0.34)	0.17	0.468	30	0.784	50

All data are expressed in percentage (%)

Average	*Robust mean of all submitted results (± two times the standard error or range with α = 0.05)*
STD	*Standard deviation of the average, calculated from the reproducibility standard deviation divided 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 (between-laboratory variability)*
RSD_R	*Relative reproducibility standard deviation*

First indication provided by the robust analysis (table 5) is that all averages are higher than the expected target values but almost similar to the means from classical statistics. This stands for a global overestimation among results that might not strictly be linked to some few aberrant values.

The reproducibility or between-laboratory variability, expressed by RSD_R, is rather poor and shows a high variation ranging from 50% to 84%. Nevertheless when comparing the present reproducibility with that from the CRL-AP ILS 2006 study, it is about twice as better. Actually in 2006 it was presenting an extremely high variation of about 100% (ranging from 85% to 116%). This hold especially true for the 1% Fish 2006 sample that allows comparison between both studies: 50% in the present study versus 116% in the former study. This improvement could partly be explained by the use of the standard calculation tool which avoids possible errors. Notwithstanding this general observed improvement on the RSD_R, there must still be important lab biases that could stand for the high standard deviations observed from the gross results. Whether those lab biases are related to *d* or *S2* values needs further investigations. Finally one can note that the highest between-laboratory variability is observed for the sample presenting the smallest level of adulteration as could be expected.

The repeatability or within-laboratory variability, expressed by RSD_r, ranges from 26% to 53%. The repeatability values are only acceptable for the three samples having the highest percentage of fish meal. A comparison of those values with that from CRL-AP ILS 2006 study, ranging from 12% to 30%, reveals nonetheless a weaker repeatability in the present study especially for the two lowest levels of fish adulteration. On the other hand the present repeatability values appears to be more logical than that from previous study where no influence of the adulteration gradation could be noted on the repeatability – on the contrary the 2006 0.25% Fish III which was the smallest percentage sample had the second best repeatability value (17%). Concerning the 1% Fish 2006 sample, it obtained exactly the same within-laboratory variability through both present and former study, namely 30%.

Z-score analysis (table 6) was realised for detecting outlying results that could impact on the rather poor observed reproducibility. Z-scores were calculated from the robust analysis figures, i.e. differences between reported values and the robust averages over the reproducibility standard deviation.

Table 6: z-Scores results. The action values are in bold red and the warning values in bold blue. No ranking was realised.

z-scores

	0.15 % Fish		0.4 % Fish		0.7 % Fish		1 % Fish		1 % Fish 2006	
Lab ID	I	II	I	II	I	II	I	II	I	II
1	-0.643	-0.908	-0.528	-0.549	0.043	-0.242	-0.536	-0.359	-0.460	-0.641
2	0.118	-0.178	-0.085	-0.417	-0.092	-0.490	-0.298	-0.696	0.637	0.907
5	-0.441	-0.078	-0.437	-0.173	-0.066	-0.624	0.181	-0.512	-0.440	0.940
6	0.239	-0.176	-0.708	-0.543	-0.757	-0.460	-0.760	-0.861	-0.160	-0.432
8	-0.310	0.612	0.358	0.559	0.233	0.481	0.107	-0.132	-0.024	0.297
10	-1.177	-1.175	-1.396	-1.429	-1.345	-1.347	-1.387	-1.400	-1.889	-1.921
11	1.484	0.111	0.422	1.125	0.294	1.350	-0.370	-0.393	0.484	1.326
12	-1.111	-1.106	-1.242	-0.100	-0.424	-0.839	-0.885	-0.756	-0.615	-1.126
13	-1.188	-1.188	-1.203	-0.580	-1.312	-0.926	-0.828	-0.090	-0.352	-0.045
14	**2.510**	-0.028	0.702	-0.033	0.468	0.986	0.794	0.494	0.032	-0.123
17	-0.842	-0.625	-0.740	0.142	-0.787	-0.437	-0.677	-0.640	-0.169	-0.341
18	1.945	**2.347**	**2.274**	**3.185**	**3.307**	1.305	**3.045**	1.636	1.464	1.793
21	**2.631**	**2.026**	**2.459**	0.988	**3.073**	1.697	**2.643**	1.931	**2.393**	0.997
22	0.289	-0.445	1.781	0.988	0.791	0.693	1.088	0.491	-0.222	1.202
23	0.084	0.076	-1.003	-0.605	-0.437	-0.307	-0.275	-1.165	-1.507	-0.380
24	-0.239	-0.969	-0.965	-1.199	-1.317	-0.974	-0.670	-0.767	-0.993	-1.080
28	0.151	0.837	-0.206	-0.201	-0.151	-0.224	-0.102	0.010	-0.785	-0.157
29	1.874	-0.742	-0.791	-0.109	-0.602	-1.148	-1.237	0.308	-0.867	0.014
30	0.168	-0.631	-0.533	-0.095	-0.638	-0.697	-0.368	-0.209	-0.480	-0.540
33	0.222	-0.581	-0.033	1.303	0.700	0.779	1.046	0.844	1.205	0.716
34	1.058	-0.827	0.091	1.342	0.839	1.286	**3.573**	1.178	-0.770	1.298
35	-0.039	1.306	0.470	**4.317**	1.384	0.562	1.385	1.034	-0.315	**2.640**

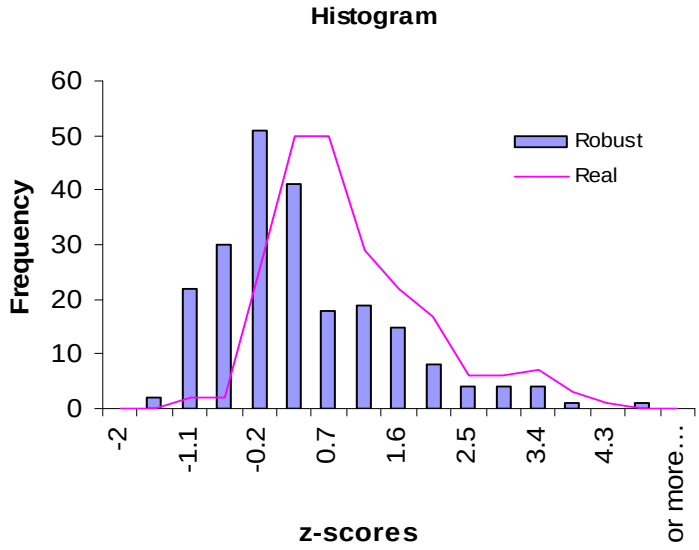
Out-of-range z-scores are limited to few labs:

- Labs 18 and 21 have the majority of warning and actions values
- Labs 34 and 35 have each an action value. In addition lab 35 presents also one warning value.
- Lab 14 has one warning value.

From table 6 it a appears also that no single sample is condensing out-of-range values of z-scores, as it was observed in the CRL-AP ILS 2006 study – the majority of aberrant z-score values were recorded in the sample with the smallest concentration of adulteration. From these z-score data, the impression of having at least two potential outliers, lab 18 and 21, is confirmed as well as the aberrant gross results of labs 34 and 35.

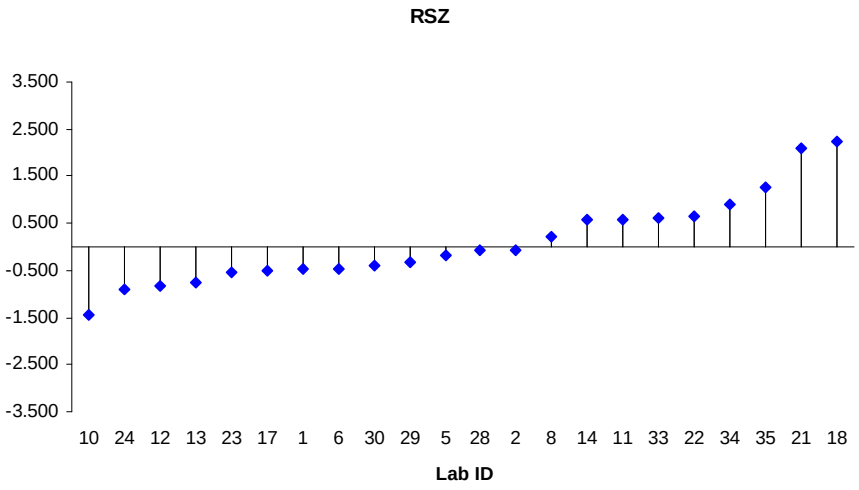
What is confirmed for the maximal gross results, does not appear to be true for the minimal gross results as reported for lab 10 for instance which had globally severe underestimations of percentages. Z-score analysis does not reveal lab 10 as an outlier although its results is questionable.

The distribution of the z-scores was analysed (graph 1 below).



Graph 1: Distribution of z-scores from robust and classical statistics.

Both z-scores distributions, the one based on robust assigned values and that on the real attended values, were analysed. Both distributions show a clear shift to the left – or left skewing – of a non Gaussian one as it was yet previously observed from former CRL-AP ILS 2006 study. Both distributions are similar i.e. long tailed reflecting a number of high overestimations on the either assigned robust averages or real expected values of percentages. But whereas the robust based z-score distribution shows the maximum of values within the -0.2 and 0.25 classes (92 values) the real data based z-score distribution presents its maximum number of frequency within the 0.25 and 0.7 classes (100 values) thus clearly revealing a global small overestimation of the calculated percentages over its expected values. In order to verify if the high number of overestimations as mentioned earlier is due to some few strong lab biases, we calculated the rescaled sum of z-score (RSZ) (graph 2).



Graph 2: RSZ or z-score global mean.

Having chosen an acceptance limit of |1|, the RSZ reveals strong positive biases for labs 18, 21 and 35 and strong negative biases for lab 10. This partly accounts for the left skewing of the distributions curves, but not for the global slight background overestimations. RSZ revelations, according to the initial choice of |1| value of RSZ within this study, have been confirmed by the relative laboratory performance (RLP) analysis as recommended by von Holst and Alder [9] (table 7).

Table 7: Relative Laboratory Performance.

Lab ID	RLP
1	0.54
2	0.48
5	0.47
6	0.57
8	0.36
10	**1.47**
11	0.89
12	0.89
13	0.89
14	0.94
17	0.59
18	**2.34**
21	**2.19**
22	0.92
23	0.74
24	0.96
28	0.39
29	0.94
30	0.48
33	0.83
34	**1.50**
35	**1.81**

for n combined z-scores = 10 ;
RLP <1.1 is a good performance
RLP >1.1 and <1.4 is a satisfactory performance
RLP >1.4 and <1.6 is a questionable performance
RLP >1.6 is unsatisfactory

If from this analysis of the performance, some labs were shown to be aberrant and thus impacting partly on the between-laboratory and within-laboratory variabilities, the global highlighted overestimation still has to be explained. We therefore analysed the different calculation parameter that could interfere with the final calculated percentages, i.e. *S2* the parameter resulting from both sedimentation and Alizarin red staining process and *d* resulting from the grid counting process.

4.2.4. Respective influence of formula terms on quantification

4.2.4.1. Sedimentation – Staining process

From the instructions, it was clearly indicated that the sedimentation had to be realised from a sample intake *W*, of 10g at 0.01g. Some participants did not respect the protocol and worked on a higher *W*: about 60 cases on the total of 220 (~30%). Nonetheless as the subsequent weight measures on *S1* and *S2* derived from *W* it must be looked at the respective ratios rather than at the proper weights. So the impact of a higher *W* can be minimized in the present interlaboratory study.

The first parameter to analyse is the amount – or percentage – of sediment obtained from the sedimentation, in other terms to study the variability of the *S1/W* ratio. Results are expressed in table 8.

Table 8: *S1/W* ratio. The upper part table gives the ratios expressed in %. The two maximal values for a sample are indicated in bold red, whereas the two minimal values are in bold blue. The lower part table provides some basic statistics (Legend: ND = not determined, STD = standard deviation, min = minimum value, max = maximum value)

***S1/W* ratio**

	0.15 % Fish		0.4 % Fish		0.7 % Fish		1 % Fish		1 % Fish I 2006	
Lab ID	I	II	I	II	I	II	I	II	I	II
1	2.61%	2.48%	2.58%	2.57%	2.49%	2.71%	2.55%	2.67%	1.02%	1.12%
2	3.26%	3.19%	3.21%	2.95%	2.74%	3.02%	3.09%	2.91%	1.06%	0.98%
5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	2.64%	2.62%	2.72%	2.50%	2.57%	2.74%	2.66%	2.64%	1.07%	0.96%
8	3.11%	3.12%	3.17%	3.11%	3.03%	2.99%	3.03%	3.19%	1.09%	0.83%
10	2.25%	2.36%	**1.85%**	2.42%	2.52%	2.51%	**1.93%**	2.83%	**0.48%**	**0.65%**
11	3.54%	3.78%	3.50%	3.44%	3.47%	3.44%	3.71%	2.68%	1.23%	1.22%
12	**3.98%**	**4.06%**	**4.44%**	**4.28%**	**4.45%**	**4.16%**	**4.40%**	**4.37%**	1.38%	**1.46%**
13	3.97%	3.24%	4.16%	3.09%	2.91%	2.94%	3.77%	3.14%	1.23%	1.00%
14	2.64%	2.10%	2.32%	2.12%	2.78%	**2.13%**	2.60%	2.20%	1.24%	0.92%
17	2.97%	3.03%	2.58%	2.81%	2.84%	2.64%	2.88%	3.54%	0.92%	0.99%
18	3.38%	3.20%	3.35%	3.15%	3.23%	3.00%	3.35%	3.25%	1.14%	1.19%
21	3.33%	3.16%	3.13%	3.53%	3.25%	3.43%	3.27%	3.14%	**2.05%**	1.21%
22	3.24%	3.26%	2.88%	2.93%	3.23%	2.94%	3.21%	3.12%	0.99%	1.17%
23	3.07%	3.30%	3.06%	2.83%	2.97%	3.06%	3.11%	3.18%	1.14%	1.08%
24	3.64%	3.31%	3.50%	ND	3.31%	3.13%	3.45%	3.41%	1.42%	1.15%
28	2.19%	2.28%	2.12%	**2.07%**	2.01%	**2.15%**	2.21%	2.27%	0.81%	0.89%
29	2.69%	2.73%	2.59%	2.80%	2.78%	2.49%	2.98%	2.92%	0.97%	0.96%
30	2.92%	3.07%	3.26%	3.38%	3.47%	2.86%	3.52%	3.14%	0.87%	0.99%
33	3.26%	3.23%	3.12%	3.61%	3.84%	3.50%	3.83%	3.31%	1.08%	1.16%
34	**2.10%**	**1.90%**	2.45%	2.10%	**2.13%**	2.20%	**2.13%**	2.37%	1.00%	1.02%
35	3.44%	3.53%	2.95%	3.11%	3.12%	3.14%	3.39%	3.71%	1.30%	1.39%

mean	3.03%	2.97%	2.96%	3.07%	1.09%
STD	0.53%	0.59%	0.52%	0.55%	0.25%
min	1.90%	1.85%	2.01%	1.93%	0.48%
max	4.06%	4.44%	4.45%	4.40%	2.05%
median	3.18%	3.06%	2.98%	3.14%	1.07%

The means of percentage of sedimentation are very stable for materials prepared with a same matrix. The standard deviations observed on those percentages obtained for each material are low. Therefore the between-laboratory variation is low and satisfying – as it is also for the repeatability (results not shown). Few exceptions are however noted:

- Lab 5 did not report any *S1* values.
- Lab 12 presents almost always the highest value of sediment percentage (9 on 10). Investigations on its sedimentation protocol implementation should be undertaken (use of TCE ?).
- Labs 10 and 34 obtain repeatedly low values of *S1/W* ratio. Although their RLP proved them to be questionable, no correlation could be found between the observed low percentages of sediment and the corresponding gross results.

The observed difference of percentage of sediment from material 1% Fish 2006 (mean of 1.09% and STD of 0.25%) is a matrix effect and is in the range of values observed for that material in CRL-AP ILS 2006 study [2].

The impact of the sedimentation process on its own is likely to be negligible.

Participants were asked to stain the sediment by the described Alizarin red staining protocol and to weigh again the dried stained sediment in order to obtain the *S2* value used by the calculation tool. Focusing on the influence of the staining on the quantity of sediment that can be used for microscopic analysis is required. This holds especially true as the staining process includes numerous steps where a potential waste of material can arise (bleaching with sodium hypochlorite, numerous rinsing with pouring off the tubes in between each steps….). This impact of the staining or waste of materials is assessed by the *S2/S1* ratio illustrated in table 9.

Table 9: *S2/S1* ratio. The upper part table gives the ratios expressed in %. The two maximal values for a sample are indicated in bold red, whereas the two minimal values are in bold blue. The lower part table provides some basic statistics (Legend: ND = not determined, STD = standard deviation, min = minimum value, max = maximum value)

***S2/S1* ratio**

	0.15 % Fish		0.4 % Fish		0.7 % Fish		1 % Fish		1 % Fish 2006	
Lab ID	I	II	I	II	I	II	I	II	I	II
1	38.31%	42.34%	48.84%	40.86%	34.54%	38.75%	44.71%	48.31%	50.00%	58.04%
2	**99.69%**	**99.70%**	**99.69%**	**99.66%**	**99.64%**	**99.67%**	**99.68%**	**99.66%**	99.22%	**98.98%**
5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	68.35%	59.18%	56.53%	62.31%	63.47%	61.61%	58.64%	66.43%	67.17%	64.85%
8	64.22%	63.58%	61.51%	61.22%	63.04%	64.12%	64.92%	64.58%	75.23%	90.36%
10	**0.44%**	**0.42%**	**1.08%**	**0.41%**	**0.79%**	**0.80%**	**1.55%**	**0.71%**	**6.25%**	**3.08%**
11	49.72%	55.03%	57.71%	56.98%	52.16%	51.16%	50.13%	75.37%	60.16%	61.48%
12	54.37%	64.23%	57.90%	60.10%	59.96%	61.35%	54.81%	59.74%	47.70%	51.53%
13	48.66%	57.06%	47.24%	59.81%	56.86%	59.60%	51.55%	62.23%	64.80%	72.28%
14	88.64%	98.10%	90.95%	95.28%	96.76%	96.73%	94.64%	97.29%	89.52%	96.74%
17	56.57%	58.09%	68.22%	59.43%	56.69%	64.02%	60.76%	59.04%	63.04%	71.72%
18	67.36%	60.03%	63.69%	64.94%	62.84%	58.46%	66.19%	63.70%	69.98%	70.68%
21	57.36%	57.91%	55.27%	53.26%	56.92%	50.44%	63.91%	57.96%	77.07%	58.68%
22	64.72%	67.89%	75.00%	76.35%	70.37%	72.20%	75.16%	73.40%	**100.00%**	81.20%
23	47.88%	40.91%	29.64%	56.89%	38.05%	42.81%	50.48%	33.96%	16.67%	38.89%
24	51.65%	56.50%	50.57%	ND	44.41%	48.56%	48.70%	52.20%	50.70%	51.30%
28	71.00%	70.99%	71.05%	71.26%	70.19%	70.23%	71.28%	72.18%	74.45%	69.83%
29	69.85%	74.64%	77.57%	71.38%	69.61%	75.10%	68.87%	74.15%	69.39%	79.59%
30	55.60%	51.29%	53.68%	53.28%	52.46%	53.51%	55.82%	55.66%	60.96%	62.44%
33	56.75%	54.49%	56.09%	52.91%	51.04%	48.00%	50.91%	58.31%	65.74%	62.93%
34	98.58%	97.91%	97.55%	97.16%	97.66%	98.19%	98.12%	97.89%	95.00%	94.17%
35	61.34%	59.49%	61.69%	58.20%	58.97%	58.28%	57.82%	73.05%	57.69%	61.15%

mean	60.97%	61.78%	60.24%	62.73%	65.73%
STD	20.99%	21.36%	21.77%	21.20%	22.18%
min	0.42%	0.41%	0.79%	0.71%	3.08%
max	99.70%	99.69%	99.67%	99.68%	100.00%

From table 9, we observe that the staining is responsible for a waste of material of about 38%. This rate of sediment lost appears to be constant, although a probable matrix effect is observed for the 1% Fish 2006 samples where a few more sediment is recovered after staining. This could result from a higher content of less soluble elements in the matrix used for that material but this has to be verified. In general, the reproducibility is acceptable and the repeatability (not shown) is good too. Nevertheless some exceptions are noted:

- Labs 2, 14 and 34 have repeated abnormally high percentages of sediment after staining. Whether those participants actually realised the staining properly according the protocol has to be raised. Maybe they did not make the staining at all. Another possibility might be that of a weighing of *S2* after incomplete drying.
- Lab 10 looses too much sediment from the staining. The implementation of the protocol such as the carefully tube's pouring off is questionable. This lab presents the lowest gross results among all participants as noted from table 4. This might be explained by the fact that after the staining they have too small amount of sediment material to make a correct quantification.
- Lab 22 has a recovery rate of 100% for one replicate.

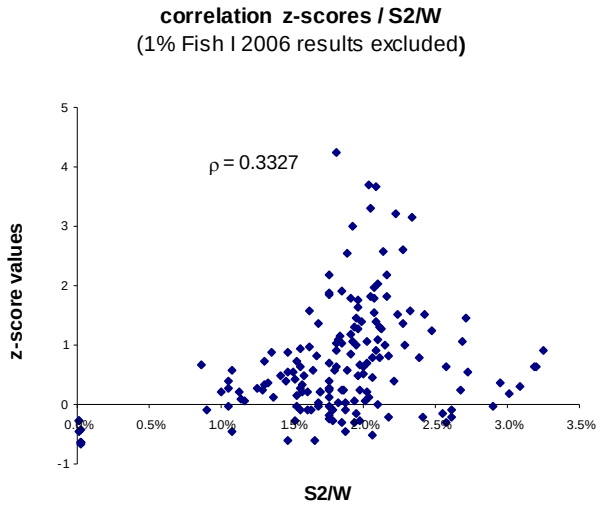
No correlations could be detected between the *S2/S1* ratios and both gross results and z-scores.

The final influence of the combined sedimentation – staining process is evaluated by the *S2/W* ratio presented in tables 10 and 11 (next page).

On the exception of some few outlying S2/W ratios such as for labs 2 and 10 (logically derived from preceding observations on the S2/S1 ratio table), the reproducibility is good. Same mention can be done concerning the repeatability which is revealed to be excellent.

The matrix used for the 1% Fish 2006 material yields a final amount of stained sediment that is about 40% lower than that observed for the other matrix used for all other materials.

Investigations on possible correlations existing between the S2/W ratio and the calculated gross results or the z-scores failed to demonstrate any cause-effect link. The type of graph obtained is illustrated hereunder.



Graph 3: Correlation graph between z-scores and the S2/W ratio. (Legend: ρ = Pearson correlation coefficient).

From graph 3 a Gaussian distribution of the S2/W can be suspected. Actually the distribution analysis of those values revealed an almost perfect narrow bell shaped distribution (not illustrated) according to the observed very good reproducibility and repeatability.

Table 10: *S2/W* ratio. The upper part table gives the ratios expressed in %. The two maximal values for a sample are indicated in bold red, whereas the two minimal values are in bold blue. The lower part table provides some basic statistics (Legend: STD = standard deviation, min = minimum value, max = maximum value)

***S2/W* ratio**

	0.15 % Fish		0.4 % Fish		0.7 % Fish		1 % Fish		1 % Fish I 2006	
Lab ID	I	II	I	II	I	II	I	II	I	II
1	1.00%	1.05%	1.26%	1.05%	0.86%	1.05%	1.14%	1.29%	0.51%	0.65%
2	**3.25%**	**3.18%**	**3.20%**	**2.94%**	**2.73%**	**3.01%**	**3.08%**	**2.90%**	1.05%	0.97%
5	1.45%	1.30%	1.33%	1.08%	1.80%	1.17%	1.53%	1.37%	0.54%	0.90%
6	1.80%	1.55%	1.54%	1.56%	1.63%	1.69%	1.56%	1.75%	0.72%	0.62%
8	2.00%	1.99%	1.95%	1.90%	1.91%	1.92%	1.97%	2.06%	0.82%	0.75%
10	**0.01%**	**0.01%**	**0.02%**	**0.01%**	**0.02%**	**0.02%**	**0.03%**	**0.02%**	**0.03%**	**0.02%**
11	1.76%	2.08%	2.02%	1.96%	1.81%	1.76%	1.86%	2.02%	0.74%	0.75%
12	2.17%	2.61%	2.57%	2.57%	2.67%	2.55%	2.41%	2.61%	0.66%	0.75%
13	1.93%	1.85%	1.97%	1.85%	1.66%	1.75%	1.94%	1.95%	0.80%	0.72%
14	2.34%	2.06%	2.11%	2.02%	2.69%	2.06%	2.47%	2.14%	**1.11%**	0.89%
17	1.68%	1.76%	1.76%	1.67%	1.61%	1.69%	1.75%	2.09%	0.58%	0.71%
18	2.27%	1.92%	2.13%	2.05%	2.03%	1.75%	2.22%	2.07%	0.80%	0.84%
21	1.91%	1.83%	1.73%	1.88%	1.85%	1.73%	2.09%	1.82%	**1.58%**	0.71%
22	2.10%	2.21%	2.16%	2.24%	2.27%	2.12%	2.42%	2.29%	0.99%	0.95%
23	1.47%	1.35%	0.91%	1.61%	1.13%	1.31%	1.57%	1.08%	0.19%	0.42%
24	1.88%	1.87%	1.77%	1.78%	1.47%	1.52%	1.68%	1.78%	0.72%	0.59%
28	1.55%	1.62%	1.51%	1.47%	1.41%	1.51%	1.58%	1.64%	0.61%	0.62%
29	1.88%	2.04%	2.01%	2.00%	1.94%	1.87%	2.06%	2.16%	0.67%	0.77%
30	1.62%	1.57%	1.75%	1.80%	1.82%	1.53%	1.97%	1.75%	0.53%	0.62%
33	1.85%	1.76%	1.75%	1.91%	1.96%	1.68%	1.95%	1.93%	0.71%	0.73%
34	2.07%	1.86%	2.39%	2.04%	2.08%	2.16%	2.09%	2.32%	0.95%	0.97%
35	2.11%	2.10%	1.82%	1.81%	1.84%	1.83%	1.96%	2.71%	0.75%	0.85%

mean	1.81%	1.79%	1.75%	1.89%	0.72%
STD	0.59%	0.59%	0.58%	0.59%	0.26%
min	0.01%	0.01%	0.02%	0.02%	0.02%
max	3.25%	3.20%	3.01%	3.08%	1.58%
median	1.87%	1.83%	1.78%	1.96%	0.73%

Table 11: Robust statistics results on *S2/W* ratio. (Legend: see table 5)

	Average	STD	s_r	RSD_r	s_R	RSD_R
0,15% Fish	1.84 (± 0.16)	0.08	0.117	6	0.361	20
0,4% Fish	1.83 (± 0.16)	0.08	0.093	5	0.393	22
0.7% Fish	1.77 (± 0.18)	0.09	0.139	8	0.411	23
1% Fish	1.93 (± 0.18)	0.09	0.160	8	0.427	22
1% Fish 2006	0.73 (± 0.08)	0.04	0.086	12	0.183	25

all data are expressed in percentage (%)

As a conclusion the combined sedimentation – Alizarin red staining protocol has only a very limited or almost non significant influence on the quantification in the present study. However this holds true provided that a feed matrix does not contain excessive amounts of hydrosoluble elements, such as certain salts, and that the operator is working according the art-of-state in order to avoid disproportionate waste of sediment material. This conclusion is in line with that from the previous CRL-AP ILS 2006 study which stated that the sedimentation had probably only minor influence on the quantification of animal proteins in feed by the EC 126/2003 directive method.

4.2.4.2. *d* Factor

This factor for the estimated portion of fish particles in the sediment might influence the results as, although being automatically calculated by the calculation tool, it actually reflects the identification and grid counting abilities of each microscopist. For reminder this study aims also at filling the hiatus of the EC 126/2003 directive which consists of the absence of any definition for this factor and consequently the inability to quantify it. The present proposed formula for *d* (see 4.2.1.) depends on the number of grid counts on the different types of particles that can be classified either as fish or other. The grid counting principle is the correct application of the stereology method [10, 11] for taking into consideration the volumes of the particles instead of their sole number.

A first analytical overview of the reported *d* values yet provides valuable information (table 12, next page):

There is a lot of variation among *d* values. The standard deviations which are almost equivalent to the means indicate a very high variability between the results. Interestingly when looking at the maxima delivered by some labs, one can note that those labs are roughly the same as those presenting systematically maximal values for the gross results:

- Labs 18 and 21 have repeated maxima (4/10 and 3/10 respectively)
- Same rules for the maxima of labs 34 and 35

This is less obvious for the minima: labs 12, 13 and 24 present numerous low values of *d*, their calculated gross results are also very low but not the lowest, the later being attributed to lab 10. Concerning this participant precisely the *d* values of lab 10 appears conversely rather high.

Cause of this heterogeneity is unclear, either poor reproducibility or repeatability.

Robust statistics (table 13, next page) were applied in order to take the suspected aberrant values into consideration and to check for outliers.

Robust averages are very close to the means and medians, thus neither general overestimation nor underestimation can be detected but on the contrary this might reflect a globally clumped distribution of the estimates of *d* values for all sample materials.

This is confirmed by the good values of within-laboratory variability, RSD_r, which has good values i.e. below 30% - at least for the 0.7% and 1% adulterated materials. Interestingly the observed values for the repeatability of *d* matched almost the repeatability values of the gross results from table 5. This might indicate that *d* is likely the sole source of variability, at least concerning the within-laboratory variations, through this study. This is supported by our conclusions on the weak impact of the sedimentation – staining

Table 12: *d* correction factors. The upper part table gives the ratios expressed in %. The two maximal values for a sample are indicated in bold red, whereas the two minimal values are in bold blue. The lower part table provides some basic statistics (Legend: STD = standard deviation, min = minimum value, max = maximum value)

***d* factor**

	0.15 % Fish		0.4 % Fish		0.7 % Fish		1 % Fish		1 % Fish 2006	
Lab ID	I	II	I	II	I	II	I	II	I	II
1	3.00%	1.00%	5.00%	6.00%	16.00%	11.00%	10.00%	11.00%	23.00%	16.00%
2	2.00%	2.00%	3.00%	2.00%	5.00%	3.00%	5.00%	**3.00%**	20.00%	23.00%
5	2.00%	4.00%	8.00%	5.00%	7.00%	6.00%	13.00%	9.00%	25.00%	23.00%
6	4.00%	3.00%	3.00%	4.00%	4.00%	5.00%	5.00%	4.00%	20.00%	20.00%
8	2.00%	4.00%	7.00%	8.00%	8.00%	10.00%	10.00%	8.00%	19.00%	24.00%
10	5.00%	6.00%	15.00%	6.00%	14.00%	13.00%	17.00%	17.00%	26.00%	26.00%
11	7.00%	3.00%	7.00%	9.00%	9.00%	15.00%	7.00%	7.00%	26.00%	35.00%
12	0.20%	**0.10%**	**0.50%**	4.00%	4.00%	2.00%	**3.00%**	**3.00%**	16.00%	**9.00%**
13	**0.00%**	**0.00%**	**1.00%**	3.00%	**0.00%**	3.00%	4.00%	9.00%	16.00%	21.00%
14	8.00%	3.00%	7.00%	5.00%	7.00%	11.00%	12.00%	11.00%	14.00%	16.00%
17	1.00%	2.00%	3.00%	7.00%	4.00%	6.00%	5.00%	5.00%	25.00%	18.00%
18	7.00%	9.00%	12.00%	**16.00%**	**23.00%**	15.00%	**26.00%**	19.00%	34.00%	**35.00%**
21	**10.00%**	8.00%	**16.00%**	9.00%	**24.00%**	18.00%	24.00%	24.00%	22.00%	33.00%
22	3.00%	2.00%	11.00%	8.00%	10.00%	10.00%	13.00%	11.00%	14.00%	26.00%
23	4.00%	4.00%	3.00%	4.00%	8.00%	8.00%	9.00%	**3.00%**	20.00%	30.00%
24	2.00%	**20.00%**	2.00%	**1.00%**	**0.00%**	3.00%	6.00%	5.00%	**11.00%**	12.00%
28	4.00%	6.00%	6.00%	6.00%	9.00%	8.00%	11.00%	11.00%	16.00%	23.00%
29	8.00%	1.00%	2.00%	5.00%	4.00%	**1.00%**	**1.00%**	10.00%	13.00%	20.00%
30	4.00%	2.00%	4.00%	5.00%	4.00%	4.00%	7.00%	9.00%	22.00%	18.00%
33	4.00%	2.00%	6.00%	10.00%	11.00%	13.00%	16.00%	15.00%	**35.00%**	29.00%
34	5.00%	1.00%	5.00%	9.00%	11.00%	12.00%	**31.00%**	14.00%	10.00%	27.00%
35	3.00%	6.00%	8.00%	**23.00%**	15.00%	11.00%	18.00%	12.00%	17.00%	**43.00%**

mean	4.03%	6.58%	8.75%	10.75%	22.07%
STD	3.53%	4.51%	5.58%	6.72%	7.55%
Min	0.00%	0.50%	0.00%	1.00%	9.00%
max	20.00%	23.00%	24.00%	31.00%	43.00%
median	**3.00%**	**6.00%**	**8.00%**	**10.00%**	**21.50%**

Table 13: Robust statistics results on *d* factor. (Legend: see table 5)

	Average	STD	s_r	RSD_r	s_R	RSD_R
0,15% Fish	3.64 (± 1.16)	0.58	1.926	53	2.716	75
0,4% Fish	6.00 (± 1.50)	0.75	2.426	40	3.531	59
0.7% Fish	8.41 (± 2.30)	1.15	2.272	27	5.403	64
1% Fish	10.09 (± 2.54)	1.27	2.252	22	5.960	59
1% Fish 2006	21.71 (± 3.20)	1.60	5.669	26	7.507	35

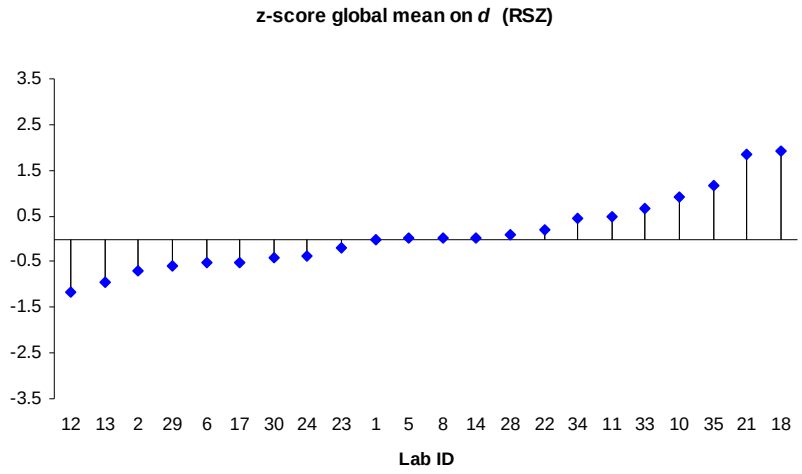
all data are expressed in percentage (%)

process on the results. Concerning the reproducibility, the values of RSD_R are comparable to those from the gross results (table 5). The reproducibility is even more always ~10% better for all materials. This would suggest that the other parameter, *S2,* accounts for a method bias of only 10% over the global reproducibility. This however should be further considered.

Z-score analyses on *d* (not shown) indicated the following action and warning values:

- Action signals : one for labs 24, 34 and 35
- Warning signals : one for labs 10 and 35, three for lab 18 and five for lab 21

The RSZ analyse, with the same acceptance limit of |1| as defined for the RSZ on the calculated percentages, confirms the strong positive biases for labs 18, 21 and 35 and strong negative biases for lab 12. All other participants present acceptable RSZ values evenly distributed around the averages.



Graph 4: RSZ or z-score global mean on *d*.

Those analyses however still lack to provide any information on the possible reasons for the global overestimations of calculated percentages or gross results. In order to get further insights on this issue and as *d* relies on the number of counts on the correctly identified particles, the source of variability for *d* was investigated based on the participant's provided data such as the number of counts from the 20 fields observed as stated in the instructions.

In the study, the instructions given to the participants were to realise the grid counting on 5 randomly chosen fields per slides. This had to be performed on 2 slides from both stained sediment fractions (<250µ and >250µ) and 5 fields per slide have to be counted, in other terms a total of 20 fields per sample had to be observed in line with the grid counting principles and in order to work on a representative portions of particles. In addition in order to achieve a sufficient accuracy, it was recommended to respect the "25 counts rule" which is the number of counts that is theoretically enough to provide a counting imprecision of about 4.5% (cf Annex 5)[‡]

Over the study, the total count number per quantification on each replicate had a grand mean value of 697 counts over the 20 fields, in other term on the average 35 counts per fields. Thus the good practice "25 counts rule" has been overall respected. This signifies also that the overall counting imprecision through the study was also decreased to 3.8% which is actually to some extent better than initially foreseen.

[‡] According to the mathematics a lower imprecision is achievable with that same grid type: a 2% imprecision would require a mean of 50 counts per field over a total of 50 randomised fields.

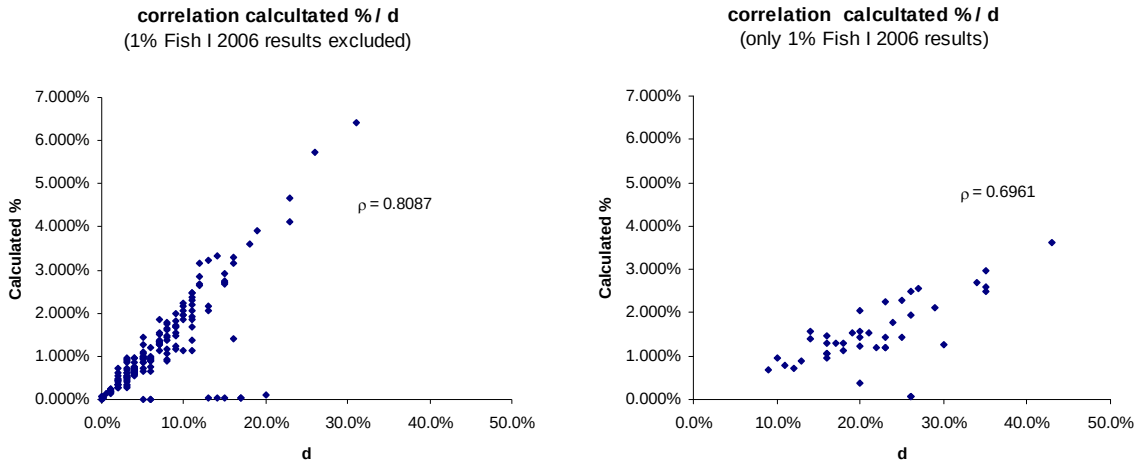
However some participants deviated from this rule:

- Labs 2, 17 and 28 have counted more hits, from 974 to 1110 mean total counts, thus obtaining a mean number of counts per field ranging from 49 to 56.
- Labs 10 and 34 presented the smallest values of mean total counts, respectively 159 and 398, thus obtaining a mean number of counts per field ranging from 8 to 20.

From the labs that counted more hits, which probably reflects slides with more sediment materials, it can be observed that their results are good, but not that better when compared to other labs, e.g. labs 8 or 30, that obtained the best relative laboratory performances (RLP) but having counted around 610 to 670 mean total counts. Concerning the labs that did not counted enough particle counts, they presented questionable RLPs. Their rescaled sum of z-scores went however in opposite direction reflecting two different causes for their deviating results:

- Lab 10 failed to analyse sufficient amount of stained sediment as it lost a lot of material during the sedimentation and staining process.
- Lab 34 presented frequent low value of *S1/W* ration and abnormally high *S2/S1* ratio (of about 100% through all sample replicates). Its sedimentation process certainly needs to be investigated. Another explanation might also possibly be that of having prepared slides with simply not enough stained sediment. A clear explanation can not be found for that lab, especially when considering the high variability of *d* values as for the replicates of the two 1% Fish materials.

In this study no correlations could be detected between the number of total counts and the calculated percentages or z-scores (except of the sole lab 10 values). It seems like the influence of the number of total counts might therefore be minor too. Since *d* is directly derived from the counting process, we looked for possible correlation between this factor and the z-scores, no one could be found. However, when we tracked for possible connection between *d* and the calculated percentage as provided by the gross results we found correlations for each used matrix:



Graph 5: Correlation graph between the calculated % and *d*. Separated correlations are calculated according to the two matrices used in the present study. (Legend: ρ = Pearson correlation coefficient).

Those correlations confirm the straight influence of *d* on the final calculation result, especially as the study demonstrates the poor impact of the sedimentation and the staining process. This is particularly evident for the results from the principal matrix of this study used for the 0.15%, 0.40%, 0.7% and 1% Fish materials where a ρ of 0.8087 is noted. The ρ of 0.6961 found for the 1% Fish 2006, prepared with the CRL-AP ILS

2006 feed matrix, is lower. One could partly explain this by the fact that only 44 results are used for its estimation, but the main cause is more related to the different matrices used. Indeed, the *S2/W* ratio for the 1% Fish 2006 material is about 2.5 times lower of that for the other materials prepared with the other feed matrix. About the same proportion is yet noted for the respective *S1/W* ratios. Logically, this means that for a same level of fish adulteration relatively more fish particles are expected to be present in the sediment of the material providing less particles from matrix origin compared to the other. This assumption on the relative concentrations of a certain particle type is verified in the present case: the *d* found for the 1% Fish 2006 is about effectively 2.2 times higher as that of the other 1% Fish material. Bridging this higher value of *d* and the lower *S2/W* ratio by using the calculation formula explains, for a fixed *f* factor, the lower slope of linear regression as it can be computed from the correlation graph for 1% Fish 2006.

4.2.5. On the general overestimation of calculated percentages

All preceding analyses lack to explain the probable origin of the generalised overestimation of results, of about twice the attended values, which was observed throughout this interlaboratory study and summarized here below:

	0.15 % Fish	0.4 % Fish	0.7 % Fish	1 % Fish	1 % Fish 2006
mean	0.618%	1.136%	1.457%	1.986%	1.584%

A first parameter that could explain the overestimations is the fixed value of *f* factor used in this study. According to the CRL-AP first workshop consensus on that point, it was decided to use a f of 0.10. If we had used the real *f* value of 0.12 as calculated for the Fish I meal before staining [2], used for both CRL-AP ILS 2006 and present study, the results would simply have been somewhat lower but still overestimated (for instance 1.214% instead of 1.457% for the 0.7% Fish). In order terms, the *f* value in the present case has almost no influence on the overestimations which would still have been observed. The key is elsewhere.

As analyses on the sedimentation – staining process demonstrated to have almost no impact or at least a weak influence on the calculated percentages, focusing on *d* is again required. It seems likely that the major source of overestimated gross results is due to a generalised overestimation of *d*. From the data collected and the realised analysis, no concluding explanations can be proposed. Only possible tracks have to be considered in order to try to solve this remaining crucial issue. Those are submitted in the next paragraphs.

4.2.5.1. Heterogeneity among slides

Slides prepared from both stained sediment fractions (<250µ and >250µ) could have been realised with rather variable sub-sampling material from the respective stained sediment batches. Although the sieving of this sediment yet segregates the small and big size particles of all nature, it does not allow insuring homogeneity among particles of animal origin (in our case fish bones and scales mainly) and particles from other origin contained in the used matrices. It is known that minerals – e.g. silicates, oxides or sulfides – have a higher density than carbonates or phosphates, which in turn are “heavier” than organic material such as bones or scales. This explains the always observed gravitational segregation of particles within a vial containing sediment. For that reason, it is almost impossible to ensure the perfect homogeneity of a slide when some sediment material is taken by a spatula, a spoon or even directly poured off material: the lightest particles being organic, the probability of having them in relative larger concentrations than their actual ones inside the sediment batch is real. The sole possibility to solve this issue is to analyse the whole sediment, which would tremendously increase the time of analysis.

Heterogeneity among slides has also to deal with heterogeneity of particle distribution on the slide itself. When covering the slide after having putted the mounting medium (i.e. glycerol) and the small quantity of sediment, during the laying down of the coverslip a migration of the smallest particles often happens. This results in heterogeneous distribution of particles. Insufficient moderate stirring of the sediment material

within the mounting medium provokes that same heterogeneity. This drawback can nevertheless be avoided by either a more rigorous and precautionary slide preparation or multiple real randomized field observations.

Further insights on how a possible in-between slide variation is required. This impact could be deduced from a study where several participants would be asked to realise quantifications on a same set of permanent slides presenting same homogeneity features, so that only the randomization and the microscopist skills could be the sources of variability.

4.2.5.2. Randomization of fields selection

Ideally an effective objective randomization of fields within a slide should be achieved for grid counting purposes. This is seldom the case. Actually, although it was explained in the protocol, we can guess that some operators didn't entirely rely on this concept – especially when the organiser highlighted that counting "nothing" or an empty field did not make sense. There might be a natural trend to "choose" or to focus on fields containing at least one fish particle because the aim of the study was announced as being a quantification exercise on fish adulterated material. Operators could have been concerned by the fact that they were not allowed to miss too much fish particles therefore leading to an involuntary overcounting of those particles. We experienced this within the CRL-AP team during the preliminary study, which leads to calculated percentage values high beyond the expected ones.

A same kind of psychological impact is also that of having underestimated the particles of other type: too much attention was drawn on the fish particles taking into consideration the objective of the study.

4.2.5.3. Alizarin red staining

The Alizarin red staining of the sediment could also partly explain the general overestimation. Although it was explicitly recommended to pay attention to other typical features of fish particles before ascertaining a spicule is truly from fish origin, we can suppose that some red coloured particles have been considered as fish ones whether they actually were not. Alizarin red has to be considered as a help for a better screening of slides and a facilitated initial detection of bones. Even though it might today be considered as the most specific staining for bones, the dye is a chelator of calcium. Consequently it stains also other type of calcium containing materials, e.g. tricalciumphosphate particles, but lacks for instance to stain others (calcium carbonate or aragonite). In the same way, the Alizarin red staining intensity is not a criterion of distinction for bone particles as the intensity is depending on the accessibility to the dye and thus possibly ranges from one particle to another depending on its size, shape, density and cleanness. Histochemistry is not an exact science and will never replace the diagnostic of a confirmed microscopist, it only helps him.

4.2.5.4. Human skills and experience

The ability of the operator for making the distinction between animal particles and particles from other origin is the keystone for the quantification because *d* depends mostly on this ability. Results from labs 18 and 21 which had the highest number of maximal values of *d* compared to the other participants might be interpreted in this direction, and thus might originate from to many false positive fish identifications. Similarly the case of the two abnormally high values of *d* from lab 35 for one of the two replicates from 0.4% Fish and 1% Fish 2006 testifies a poor repeatability possibly linked to misidentification – or analyses realised by two different persons.

The correct Köhler illumination and use of the microscope is almost as critical as the recognition skills of the operator. This aspect was highlighted in the PowerPoint protocol version as well as in the text version. A correct focussing limits the risk of overestimating particles towards others especially when grid counting is realised. From multiple past training sessions, it is obvious that this aspect related to the optical physics is seldom well understood and almost never applied. One will never enough insist on this issue which can lead to erroneous estimations.

Finally when considering the human factors and the importance of having experienced microscopists, it is relevant to mention that from the 5 participants that had either questionable of unsatisfactory relative laboratory performances (table 7) 3 of them had no former experience in quantification and did not realised the quantitative analysis from CRL-AP ILS 2006. This is not a casual fact but a complementary evidence for the weight of training.

4.2.5.5. Other possible sources

The within-laboratory variability is slightly higher in the present study than in the former. This could partly be linked to the fact that for this study, participants had to report figures without any rounding while it was allowed in CRL-AP ILS 2006 study. Actually in the later study 6 labs on the 17 that made the quantification exercise delivered rounded results. Although this probably has influenced the repeatability and the reproducibility, this has not been investigated.

The number of fields observed could be increased in order to gain in precision of counting, as by increasing the later we assume to obtain a more representative population of the stained sediment particles. Even though 20 fields from 4 slides have been considered as enough, in order to gain significantly in precision we could increase the number of fields. Nevertheless the resulting extra charge of work would probably not be acceptable for routine quantifications as requested from the European Commission. This still has to be discussed and a balance between pros and cons has to be made.

5. Conclusions

From the present study results the proposed revised protocol for quantification can not be validated.

The results still demonstrate a variability that is too high. The reproducibility although being quite improved compared to the CRL-AP ILS 2006 study can still be progressed. This allegation bases on the fact that some NRLs still present abnormal results. As long as participants to this type of test can be considered as outliers enhancements are required. The repeatability is acceptable but can be improved too.

Concerning the method itself, the present interlaboratory study showed that the proposed enhancements could be implemented by all participants. This is a major step forwards since it was previously not possible based on the sole EC 126/2003 directive because of a lack of detailed information – for reminder one NRL on three was unable to apply the quantification method as such in 2006.

The proposed improvements included mainly (1) the grid counting implementation on which *d* is calculated by mean of a clearly defined formula and (2) the use of a standardized calculation worksheet developed by the CRL-AP. Other changes were (3) the use of the Alizarin red staining and (4) the input of the dried stained sediment weight, *S2* in the formula.

The grid counting is suitable for the calculation of *d* because it makes all measurements standard (same grid and unit surface of counting). Same can be concluded from the calculation tool. However a generalized overestimation of calculated percentages was observed. The exact causes for this are still unclear. Possible explanations might be related to the heterogeneity of slide preparations, the insufficient randomisation for the field selections, the insufficient number of fields observed, a side effect of the Alizarin red staining and human factors such as the ability to identify correctly the origin of particles.

Ways of potential improvements go in the following directions: human skills development and training on quantification coupled with an increased number of fields for the grid counting. Other tracks for improvement needs further discussions among NRLs.

The interlaboratory study showed once again that, on very few exceptions, the sedimentation process and the staining are correctly implemented and consequently only have a limited impact. On the contrary, the correct evaluation of d was demonstrated to be critical as it entirely rely on the microscopists' ability to characterise the origin of a particle.

Concerning this impact of the operator, once again this study highlighted the need experience: from the 5 participants that had poor results, 3 of them did not participate to the former interlaboratory study on quantification and assumed to never make any quantitative analyses. The need of continuous training and formation is crucial.

Acknowledgment

We are especially grateful to the whole technical staff of the CRL-AP and CRA-W for their help in the sample preparation and the homogeneity study. Special acknowledgement is also formulated to the NRLs that participated to this study.

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Annex 1

List of participating NRLs

Country	Institute Name
Austria	Austrian Agency for Health and Food Safety
Belgium	Federal Agency for the Safety of the Food Chain
Bulgaria	National Diagnostic Research Veterinary Medical Institute
Cyprus	Cyprus Veterinary Services
Czech republic	Central Institute of sampling and testing in Agriculture
Denmark	The Danish Plant Directorate
Estonia	Veterinary and Food Laboratory
Finland	Finnish Food Safety Authority
France	DG for Fair Trading, Consumer Affairs and Fraud Control-Laboratory Directorate Rennes
Germany	Federal Institute for Risk Assessment
Greece	Feedstuffs Control Laboratory
Hungary	Central Agricultural Office-Directorate Food and Feed Safety-Central Feed Investigation Lab.
Ireland	Department of Agriculture and Food Microscopy Laboratory - Seed Testing Station
Italy	National Reference Center for the Surveillance and Monitoring of Animal Feed
Latvia	National Diagnostic Centre of Food and veterinary Service
Lithuania	National Veterinary Laboratory
Luxemburg	Agroscope Liebefeld-Posieux Research Station (Switzerland)
Netherlands	RIKILT Institute of food safety, Wageningen UR
Poland	National Veterinary Research Institute
Portugal	Laboratorio Nacional de Investigaçao Veterinaria
Romania	Hygiene Institute of Veterinary Health
Slovakia	State veterinary and food institute
Slovenia	Veterinary Faculty-National Veterinary Institute-Unit for pathology of animal nutrition and environmental hygiene
Spain	Laboratorio arbitral agroalimentario
Sweden	National Veterinary Institute, Dept of Animal Feed
United Kingdom	Veterinary Laboratories Agency

Annex 2

Text version of the protocol

CRL-AP Interlaboratory Study 2007
- Protocol for quantification -

1. Field of application:

These conditions shall be used for the quantification of constituents of animal origin in feedingstuffs by means of classical microscopy. The instructions contained in this protocol are complementary to those described in Directive 126/2003 EC.

2. Principle:

A quantification of constituents of animal origin in feedingstuffs will be completed based on a microscopic grid counting process of animal particles and particles of other nature on slides. The slides will be realised with Alizarin Red stained sediments. Obtaining of the sediments follows Directive 126/2003 EC. Subsequently records from the grid counting process will be filled in an Excel calculation tool that will automatically calculate the percentage of animal constituents in the feed accordingly formula from point 7.2 of Directive 126/2003 EC slightly modified.

3. Materials :

3.1. Eyepiece reticle Counting Pattern NG14 from Graticules ^{TM} (sometimes referred also as G14)

3.2. Slides (classical and hollow) and coverslips (square 20x20mm)

3.3. Fine tweezers (e.g. type Dumont 5)

3.4. 250µ mesh sieve.

Other material and equipment: cf. EC 126/2003 Directive

4. Procedure :

4.1. Obtaining of sediment and staining

Identification of constituents of animal origin is realised from the concentrated sediment according to EC 126/2003 Directive with respect to the following specific instructions differing from the Directive:

- The sediment has to be obtained from 10g (at 0.01g) of the sample material (= *W*).
- The total sediment shall be allowed to dry before weighing accurately at 0.001g (= *S1*).

After having weighed the total dry sediment, the sediment shall be stained with Alizarin red according to point 6.3 of the Directive. Special attention has to be carried on the vortex handling of the tubes containing the sediment in between each step of the staining process. After vortexing allow the particles to settle before pouring off the tubes very cautiously; it is very important to avoid wasting of sediment material during the staining procedure. After the addition of at least 10 drops of Alizarin red, mix by vortexing during at least 30s in order to stain correctly. Thereafter rinse as described in the Directive.

Allow the Alizarin red stained sediment to dry totally before weighing it at 0.001g (= *S2*)

After having obtained the stained sediment weight *S2*, sieving of the sediment is realised with a 250µ mesh sieve. Store separately the two sediment fractions obtained (<250µ and >250µ fractions).

4.2. Slide preparation and mounting

Slides have to be prepared from both stained sediment fractions: 2 slides from the <250µ fraction (using classical glass slides) and 2 slides from the >250µ fraction (using hollow slides). Mounting has to be done very accurately according the following instructions:

- Poor a few drops of glycerol or other routine mounting media referred by EC 126/2003 Directive on the slide (2-3 drops for classical slides and 4-5 drops for hollow slides). Drops deposit must be done carefully in order to avoid the formation of air bubbles.
- With a spatula tip put some sediment material on the slide. Stir gently in order to spread uniformly the material over the glycerol deposit. The amount of material may not exceed one layer of particles once spread.
- Take a coverslip, hold it with fine tweezers and lay it carefully down on the material deposit: touch the slide with one side of the coverslip at an angle of *ca*. 30°. Move coverslip towards the glycerol drop. Once in contact with the glycerol, open tweezers while allowing the coverslip to lay down by capillarity, thus gently. Allow the glycerol to spread all under the coverslip surface without pushing on the coverslip (for hollow slides you may have to press very carefully on the coverslip in order to achieve this more rapidly).

4.3. Grid counting

All slides observations and related grid counting are realised on the compound microscope at a final magnification of 100x. Before starting microscopic observations be sure that the eyepiece reticle is well inserted in your eyepiece and that the grid image projection is correctly focussed for your eyes.

Grid counting has to be realised with respect to the next principles:

- The counting area is the grid square with the crosses; the remaining part of the field is not taken into consideration. Particles visible on the microscope field but located outside this area are thus excluded from counting.
- Grid counting is intended to take into consideration the volume ratio of the different types of particles over each other. This can be achieved by the “point counting” method which basic principle is very simple: every hit of a cross on a particle adds 1 to the counts of that particle type. (*e.g*. one particle can be hit by 4 crosses, thus simply count 4; some particles inside the counting area might not be hit by a cross because of their small sizes, those particles are thus logically kept out of the counting process)
- “Point counting” requires, by definition, associating to each cross a counting point (for instance a point located at all upper right angle of each cross). Signification of this point is the following: when a counting point hits a particle it adds 1 to the count of points. In this respect a cross hitting neither completely in nor out a particle has to be counted only if its associated angle, corresponding to the counting point, is truly hitting the particle, if not it has to be excluded. Once this decision of positioning this point at one angle has been taken it has to be adhered through all sets of counting.
- Since the counting point need one has to strictly examine with a correct Kölher illumination and a sharp focus on each particle limit before deciding whether or not a cross hit can be considered. This rules specifically for grid counting on hollow slides on the >250µ fraction particles. Not respecting this can lead to an overestimation bias.
- Field selection on a slide has to be randomized as far as possible: ideally the different fields should be selected blind by travelling through a slide along the X and Y axes without looking into the eyepieces. Nevertheless if a blind selection, thus randomized, results in positioning the grid on a slide area free from any particle, select a new one as it makes no sense counting nothing; actually we are concerned by the volume ratio of different types of particles over each other rather than their global absence or presence.

- As a good practice rule and in order to reach a satisfying counting precision, the average number of total particle counts (bones and others) per field calculated over all fields of all slides should be above 25. Note that this is a mean, it does not rule out allowing to have fields with fewer particle counts.

Grid counting has to be performed on 2 slides from both stained sediment fractions (<250µ and >250µ) and 5 fields per slide have to be counted, in other terms a total of 20 fields per sample have to be observed in line with the grid counting principles.

For the counting, pay attention to the exact identification of bone or fishbone/scale particles. Consider all aspects of a particle before identifying it as a bone, cartilage or scale fragment (i.e. staining, shape, presence of osteocytes or chondrocytes, typical scale features and the like).

4.4. Calculation

As indicated in point 7 of EC 126/2003 Directive, calculation is based on the presence of bone fragments or other particles of high specific weight being in the sediment (stained sediment in present protocol).

The first computation that is required is the estimation of the portion of terrestrial bones (or fish bones and scale fragments) in the stained sediment (respectively *c* or *d*). Accordingly records needed for those estimations, which are used as correction factors, are thus:

- The number of counts for terrestrial bones (*Tc*)
- The number of counts for fish bones and/or fish scales (*Fc*)
- The number of counts for particles of other nature (*Oc*).

The formulae used for estimating *c* and *d* factors are the following:

$$c = \frac{Tc}{Tc + Fc + Oc} = \frac{Tc}{\Sigma\ all\ counts}$$

$$d = \frac{Fc}{Tc + Fc + Oc} = \frac{Fc}{\Sigma\ all\ counts}$$

Once *c* and *d* computed, calculation of the estimated value of constituents of animal origin (cf. point 7.2 of Directive) can be realised using also the following records:

- The weight of the sample material used for the sedimentation (*W*)
- The weight of the *stained* sediment (*S2*)
- The correction factor for the proportion of bones in the constituents of animal origin in the sample examined (*f*). For the present test default values, as agreed among participants of the first CRL-AP Workshop, have to be used (for reminder for fish *f* = 0.10 and for terrestrial *f* = 0.40)

The formulae used for the estimated value of constituents of animal origin are the following:

$$\text{Estimated value of constituents of terrestrial animal product (\%)} = \frac{S2 \times c}{W \times f} \times 100$$

$$\text{Estimated value of constituents of fish product (\%)} = \frac{S2 \times d}{W \times f} \times 100$$

Practically all calculations have to be executed by help of the provided Excel calculation tool file (to download from the CRL-AP intranet). Instructions of use for the calculation tool are on the file worksheet itself: follow them carefully.

After input of basic records, the *c* and *d* factors are computed automatically by the calculation file. Final results, *i.e.* the estimated values of constituents of animal product expressed in %, are also calculated automatically. Visualisation of those results on the calculation tool file is conditioned by a picklist selection of the type of constituents detected.

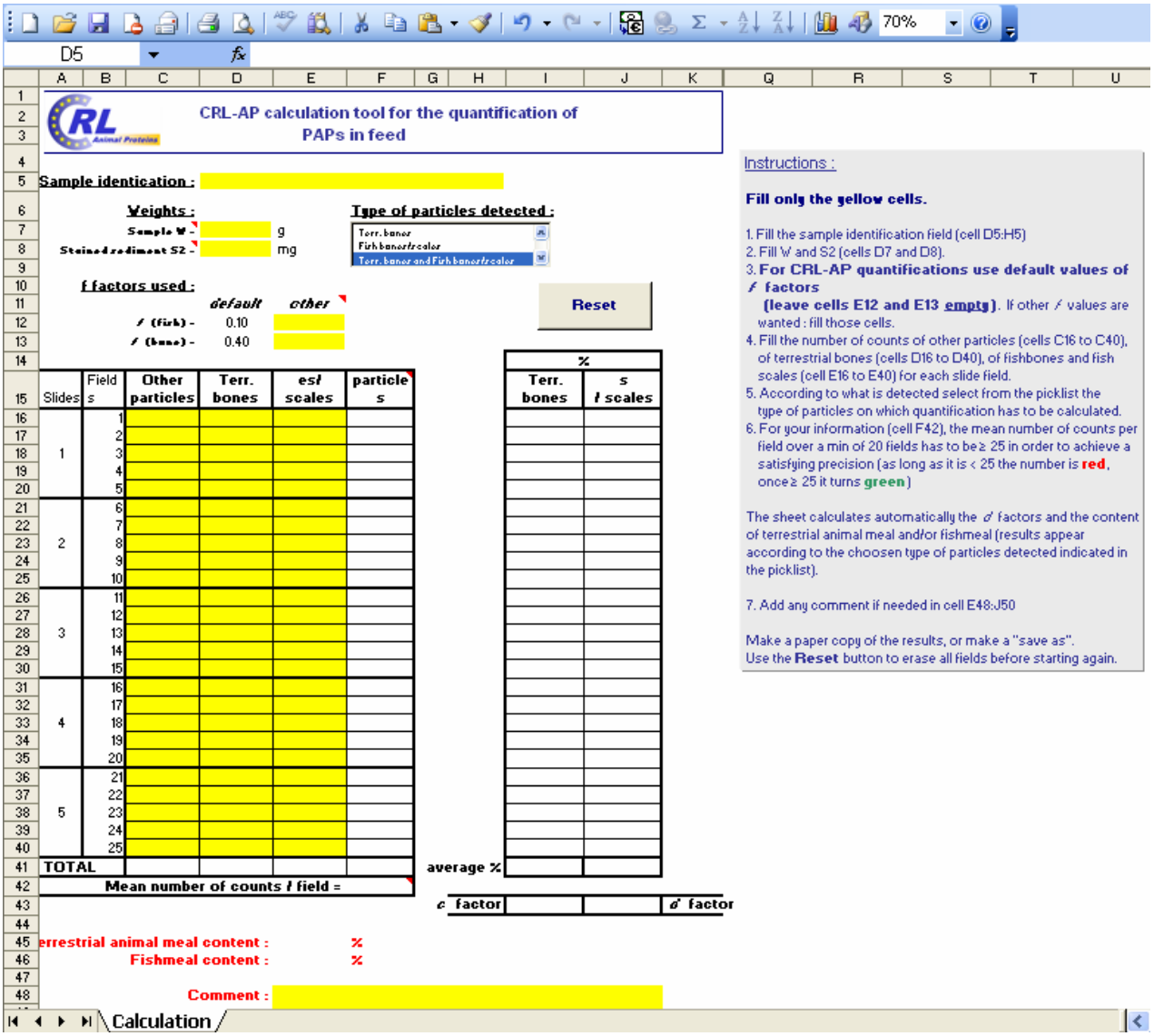
Report the asked data and obtained results on the CRL-AP Interlaboratory Study 2007 report form as they appear on the calculation tool, thus *without any rounding* of the values.

A PowerPoint slideshow of this protocol provides you needed illustrations and examples.

(to download from the CRL-AP intranet)

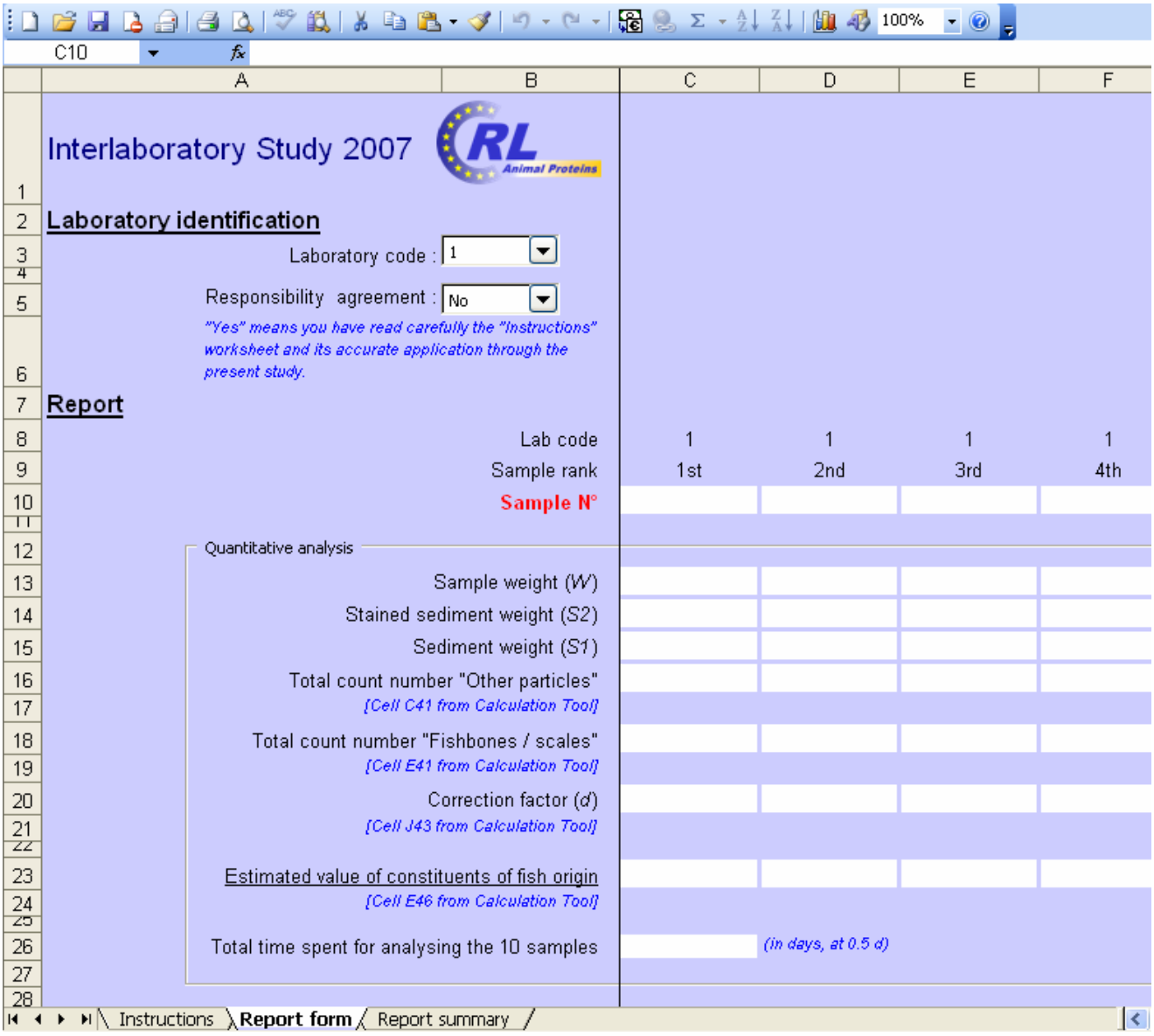
Annex 3

Excel calculation tool.



Annex 4

Excel report form.



Interlaboratory Study 2007

Laboratory identification

Laboratory code : 1

Responsibility agreement : No

"Yes" means you have read carefully the "Instructions" worksheet and its accurate application through the present study.

Report

Lab code	1	1	1	1
Sample rank	1st	2nd	3rd	4th
Sample N°				

Quantitative analysis

Sample weight (*W*)				
Stained sediment weight (S2)				
Sediment weight (*S1*)				
Total count number "Other particles" *[Cell C41 from Calculation Tool]*				
Total count number "Fishbones / scales" *[Cell E41 from Calculation Tool]*				
Correction factor (*d*) *[Cell J43 from Calculation Tool]*				
Estimated value of constituents of fish origin *[Cell E46 from Calculation Tool]*				

Total time spent for analysing the 10 samples — *(in days, at 0.5 d)*

Instructions | **Report form** | Report summary

Annex 5

Relative imprecision for grid counting.

Type of grid : NG14

Maximum number of counts per fields : 100

Theoretical % of imprecision according formula[§] : $\% = \frac{\sqrt{N_{fieds} \times \overline{m}_{counts\ /\ fields}}}{\sum counts} \times 100$

N_{fields}	Mean $_{counts\ /\ fields}$	Sum of counts	% of imprecision
20	10	200	7.07
30	10	300	5.77
40	10	400	5.00
100	10	1000	3.16
20	***25***	***500***	***4.47***
30	25	750	3.65
40	25	1000	3.16
100	25	2500	2.00
20	50	1000	3.16
30	50	1500	2.58
40	50	2000	2.24
100	50	5000	1.41

[§] As deduced from Russ J C, 2005 [10]

Annex 6

Gross results of all participants (in numerical order of lab ID)

Remark: two measures from lab 24 (in brackets in the table) were converted for the study purpose as those obviously resulted from evident encoding errors.

Laboratory identification code : **1**

	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	E	10	0,051	0,102	486	149	0,23	1,197%
2	B	10	0,126	0,258	1009	55	0,05	0,651%
3	C	10	0,086	0,249	679	134	0,16	1,417%
4	C	10	0,105	0,271	720	87	0,11	1,132%
5	D	10	0,114	0,255	665	74	0,1	1,142%
6	A	10	0,1	0,261	709	19	0,03	0,261%
7	A	10	0,105	0,248	696	9	0,01	0,134%
8	B	10	0,105	0,257	791	51	0,06	0,636%
9	D	10	0,129	0,267	624	74	0,11	1,368%
10	E	10	0,065	0,112	630	122	0,16	1,055%

Total time for the analysis of the 10 samples : **4,5**

Laboratory identification code : **2**

	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	B	10	0,32	0,321	737	23	0,03	0,968%
2	C	10,014	0,273	0,274	750	37	0,05	1,282%
3	A	10	0,325	0,326	1069	21	0,02	0,626%
4	E	12,209	0,128	0,129	910	222	0,2	2,056%
5	D	10,063	0,31	0,311	974	48	0,05	1,447%
6	B	10,027	0,295	0,296	982	25	0,02	0,730%
7	A	10,403	0,331	0,332	1359	21	0,02	0,484%
8	C	10,031	0,302	0,303	1190	36	0,03	0,884%
9	E	10,029	0,097	0,098	1169	358	0,23	2,268%
10	D	10,002	0,29	0,291	1138	38	0,03	0,937%

Total time for the analysis of the 10 samples : **15**

Laboratory identification code :	5						
Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1 D	10	0,153	0	709	110	0,13	2,060%
2 C	10	0,18	0	662	52	0,07	1,308%
3 A	10	0,145	0	748	19	0,02	0,358%
4 D	10	0,137	0	685	64	0,09	1,173%
5 A	10	0,13	0	819	35	0,04	0,532%
6 C	10	0,117	0	805	55	0,06	0,750%
7 B	10	0,108	0	764	70	0,08	0,905%
8 E	10	0,09	0	541	184	0,25	2,294%
9 E	10	0,054	0	582	170	0,23	1,212%
10 B	10	0,133	0	828	47	0,05	0,716%
Total time for the analysis of the 10 samples :	4						

Laboratory identification code :	6						
Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1 A	10	0,1801	0,2635	709	28	0,04	0,684%
2 C	10	0,163	0,2568	763	30	0,04	0,617%
3 D	10	0,1561	0,2662	569	33	0,05	0,855%
4 B	10	0,1536	0,2717	684	24	0,03	0,522%
5 E	10	0,0718	0,1069	423	105	0,2	1,432%
6 A	10	0,155	0,2619	650	21	0,03	0,485%
7 D	10	0,1751	0,2636	671	29	0,04	0,725%
8 C	10	0,1688	0,274	577	33	0,05	0,914%
9 B	10	0,1559	0,2502	561	24	0,04	0,640%
10 E	10	0,062	0,0956	437	107	0,2	1,219%
Total time for the analysis of the 10 samples :	2						

Laboratory identification code :	8						
Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1 C	10	0,191	0,303	555	51	0,08	1,607%
2 B	10	0,195	0,317	482	34	0,07	1,285%
3 A	10,05	0,201	0,313	651	14	0,02	0,421%
4 B	10,03	0,191	0,312	641	52	0,08	1,429%
5 A	10,02	0,199	0,313	528	24	0,04	0,863%
6 E	10	0,082	0,109	455	105	0,19	1,538%
7 D	10,07	0,198	0,305	603	67	0,1	1,966%
8 C	10,07	0,193	0,301	588	63	0,1	1,855%
9 D	9,99	0,206	0,319	617	54	0,08	1,659%
10 E	10,03	0,075	0,083	413	130	0,24	1,790%
Total time for the analysis of the 10 samples :	3						

Laboratory identification code :		10						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	C	10	0,002	0,252	105	17	0,14	0,028%
2	D	10	0,003	0,193	152	31	0,17	0,051%
3	B	10	0,002	0,185	92	16	0,15	0,030%
4	E	10	0,003	0,048	154	53	0,26	0,077%
5	E	10	0,002	0,065	79	28	0,26	0,052%
6	A	10	0,001	0,225	190	11	0,05	0,005%
7	D	10	0,002	0,283	97	20	0,17	0,034%
8	C	10	0,002	0,251	155	23	0,13	0,026%
9	A	10	0,001	0,236	153	10	0,06	0,006%
10	B	10	0,001	0,242	196	12	0,06	0,006%
Total time for the analysis of the 10 samples		16						

Laboratory identification code :		11						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	D	10	0,186	0,371	700	55	0,07	1,355%
2	E	10	0,074	0,123	446	158	0,26	1,936%
3	A	10	0,176	0,354	675	53	0,07	1,281%
4	B	10	0,202	0,35	652	46	0,07	1,331%
5	A	10	0,208	0,378	551	17	0,03	0,623%
6	C	10	0,181	0,347	522	53	0,09	1,668%
7	E	10	0,075	0,122	476	252	0,35	2,596%
8	C	10	0,176	0,344	584	107	0,15	2,725%
9	D	10	0,202	0,268	570	40	0,07	1,325%
10	B	10	0,196	0,344	678	70	0,09	1,834%
Total time for the analysis of the 10 samples :		10						

Laboratory identification code :		12						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	D	10,0428	0,242	0,4415	472	14	0,03	0,694%
2	A	10,0627	0,2179	0,4008	582	1	0,002	0,037%
3	C	10,0494	0,268	0,447	405	15	0,04	0,950%
4	E	10,0604	0,0663	0,139	477	93	0,16	1,075%
5	B	10,0567	0,2587	0,4468	550	3	0,005	0,140%
6	B	10,0808	0,2592	0,4313	595	23	0,04	0,957%
7	D	10,0156	0,2612	0,4372	645	22	0,03	0,860%
8	E	10,0382	0,0756	0,1467	528	52	0,09	0,675%
9	A	10,0753	0,2625	0,4087	674	1	0,001	0,039%
10	C	10,0477	0,2562	0,4176	560	12	0,02	0,535%
Total time for the analysis of the 10 samples :		3						

	Laboratory identification code :	13						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	E	10,136	0,081	0,125	655	125	0,16	1,281%
2	A	10,295	0,199	0,409	842	0	0	0,000%
3	D	10,24	0,199	0,386	827	34	0,04	0,767%
4	B	10,423	0,205	0,434	1042	9	0,01	0,168%
5	C	10,507	0,174	0,306	814	3	0	0,061%
6	D	10,301	0,201	0,323	925	89	0,09	1,713%
7	E	10,119	0,073	0,101	677	181	0,21	1,522%
8	C	10,263	0,18	0,302	840	22	0,03	0,448%
9	B	10,237	0,189	0,316	785	27	0,03	0,614%
10	A	10,487	0,194	0,34	989	0	0	0,000%
	Total time for the analysis of the 10 samples :	**2**						

	Laboratory identification code :	14						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	C	10	0,269	0,278	653	48	0,07	1,842%
2	A	10,01	0,234	0,264	658	54	0,08	1,773%
3	D	10,02	0,247	0,261	758	99	0,12	2,846%
4	E	10	0,111	0,124	674	112	0,14	1,582%
5	B	10,02	0,211	0,232	740	58	0,07	1,531%
6	C	10,03	0,207	0,214	1030	133	0,11	2,360%
7	E	10	0,089	0,092	611	120	0,16	1,461%
8	D	10,03	0,215	0,221	1025	133	0,11	2,462%
9	A	10,04	0,207	0,211	867	24	0,03	0,556%
10	B	10	0,202	0,212	897	47	0,05	1,005%
	Total time for the analysis of the 10 samples :	**1,5**						

	Laboratory identification code :	17						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	D	10	0,175	0,288	964	56	0,05	0,961%
2	E	10	0,058	0,092	559	182	0,25	1,425%
3	B	10	0,176	0,258	994	29	0,03	0,499%
4	C	10	0,161	0,284	1136	43	0,04	0,587%
5	A	10	0,168	0,297	1102	11	0,01	0,166%
6	A	10	0,176	0,303	961	15	0,02	0,270%
7	D	10	0,209	0,354	1046	53	0,05	1,008%
8	C	10	0,169	0,264	920	54	0,06	0,937%
9	B	10	0,167	0,281	813	59	0,07	1,130%
10	E	10	0,071	0,099	608	135	0,18	1,290%
	Total time for the analysis of the 10 samples :	**10**						

	Laboratory identification code :	18						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	E	10	0,07985	0,1141	420	215	0,34	2,704%
2	C	10	0,2029	0,3229	570	171	0,23	4,682%
3	B	10	0,2131	0,3346	667	95	0,12	2,656%
4	A	10	0,2274	0,3376	720	51	0,07	1,502%
5	D	10	0,2218	0,3351	660	230	0,26	5,732%
6	B	10	0,2047	0,3152	716	138	0,16	3,308%
7	E	10	0,08425	0,1192	474	257	0,35	2,962%
8	A	10	0,1918	0,3195	722	70	0,09	1,695%
9	C	10	0,1751	0,2995	725	131	0,15	2,680%
10	D	10	0,2072	0,3253	676	158	0,19	3,925%
	Total time for the analysis of the 10 samples :				10			

	Laboratory identification code :	21						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	E	10	0,158	0,205	656	182	0,22	3,432%
2	D	10	0,209	0,327	513	162	0,24	5,216%
3	D	10	0,182	0,314	565	175	0,24	4,304%
4	B	10	0,173	0,313	609	117	0,16	2,788%
5	A	10	0,191	0,333	701	77	0,1	1,831%
6	C	10	0,185	0,325	417	132	0,24	4,448%
7	A	10	0,183	0,316	685	63	0,08	1,541%
8	E	10	0,071	0,121	440	216	0,33	2,338%
9	C	10	0,173	0,343	630	136	0,18	3,072%
10	B	10	0,188	0,353	914	93	0,09	1,736%
	Total time for the analysis of the 10 samples :				5			

	Laboratory identification code :	22						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	A	10,051	0,211	0,326	544	19	0,03	0,708%
2	B	10,011	0,216	0,288	544	65	0,11	2,303%
3	D	10,019	0,242	0,322	526	81	0,13	3,223%
4	E	10,055	0,1	0,1	458	74	0,14	1,383%
5	B	10,099	0,226	0,296	535	45	0,08	1,736%
6	C	10,04	0,228	0,324	522	55	0,1	2,165%
7	C	10,04	0,213	0,295	593	64	0,1	2,067%
8	D	10,016	0,229	0,312	523	63	0,11	2,458%
9	A	10,046	0,222	0,327	550	9	0,02	0,356%
10	E	10,024	0,095	0,117	391	140	0,26	2,499%
	Total time for the analysis of the 10 samples :				3			

	Laboratory identification code :	23						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	B	10,02	0,091	0,307	592	21	0.03	0,311%
2	A	10,01	0,147	0,307	739	32	0.04	0,610%
3	A	10	0,135	0,33	681	32	0.04	0,606%
4	C	10,01	0,113	0,297	696	63	0.08	0,937%
5	D	10,01	0,157	0,311	664	69	0.09	1,476%
6	D	10	0,108	0,318	687	22	0.03	0,335%
7	E	10,01	0,019	0,114	166	41	0.20	0,376%
8	C	10	0,131	0,306	609	54	0.08	1,067%
9	B	10	0,161	0,283	650	25	0.04	0,596%
10	E	10	0,042	0,108	348	149	0.30	1,259%
	Total time for the analysis of the 10 samples :	**3**						

	Laboratory identification code :	24						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	E	10g	0,072	0,142	569	69	0,11	0,779%
2	B	10	0,177	0,35	617	12	0,02	0,338%
3	B	10	0,178	0	722	7	0,01	0,171%
4	A	10	0,188	0,364	564	14	0,02	0,455%
5	C	10	0,147	0,331	522	2	0	0,056%
6	A	10	0,187	0,331	532	3	0,2	0,105%
7	E	10	0,059	0,115	540	74	0,12	0,711%
8	D	10	0,168	0,345	571	35	0,06	0,970%
9	D	10	0,178	0,341	721	36	0,05	0,846%
10	C	10	(152)	(313)	815	22	0,03	0,400%
	Total time for the analysis of the 10 samples :	**2**						

	Laboratory identification code :	28						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	D	10,0001	0,1576	0,2211	911	110	0,11	1,698%
2	C	10,0002	0,1413	0,2013	950	90	0,09	1,223%
3	B	10,0004	0,1507	0,2121	966	60	0,06	0,881%
4	E	10,0005	0,0606	0,0814	896	165	0,16	0,942%
5	D	10,0004	0,1642	0,2275	1005	127	0,11	1,842%
6	C	10,0003	0,1512	0,2153	996	82	0,08	1,150%
7	B	10,0016	0,1473	0,2067	876	56	0,06	0,885%
8	A	10,0012	0,1552	0,2186	927	40	0,04	0,642%
9	E	10,0002	0,0625	0,0895	655	195	0,23	1,434%
10	A	10,0011	0,162	0,2282	831	53	0,06	0,971%
	Total time for the analysis of the 10 samples :	**3,5**						

	Laboratory identification code :	29						
	Sample N°	***W* (g)**	***S2* (g)**	***S1* (g)**	**Total count "Other particles"**	**Total count "Fishbones / scales"**	***d***	**Estimated value of fish const.**
1	D	10,12	0,208	0,302	502	6	0,01	0,243%
2	C	10,17	0,197	0,283	601	25	0,04	0,772%
3	E	10,1	0,068	0,098	437	66	0,13	0,878%
4	D	10,07	0,218	0,294	455	52	0,1	2,223%
5	E	10,19	0,078	0,098	409	105	0,2	1,568%
6	A	10,11	0,19	0,272	483	41	0,08	1,468%
7	B	10,15	0,204	0,263	592	14	0,02	0,463%
8	B	10,1	0,202	0,283	561	28	0,05	0,951%
9	A	10,11	0,206	0,276	563	6	0,01	0,214%
10	C	10,16	0,19	0,253	494	6	0,01	0,225%
	Total time for the analysis of the 10 samples :	**3**						

	Laboratory identification code :	30						
	Sample N°	***W* (g)**	***S2* (g)**	***S1* (g)**	**Total count "Other particles"**	**Total count "Fishbones / scales"**	***d***	**Estimated value of fish const.**
1	D	10	0,1965	0,352	715	53	0,07	1,357%
2	C	10	0,1822	0,3473	594	25	0,04	0,736%
3	E	10	0,0531	0,0871	437	125	0,22	1,181%
4	B	10	0,175	0,326	703	27	0,04	0,647%
5	B	10	0,1801	0,338	639	36	0,05	0,961%
6	A	10	0,1623	0,2919	743	31	0,04	0,650%
7	E	10	0,0615	0,0985	438	99	0,18	1,134%
8	A	10	0,1574	0,3069	695	12	0,02	0,267%
9	C	10	0,1532	0,2863	671	31	0,04	0,677%
10	D	10	0,175	0,3144	623	61	0,09	1,561%
	Total time for the analysis of the 10 samples :	**3**						

	Laboratory identification code :	33						
	Sample N°	***W* (g)**	***S2* (g)**	***S1* (g)**	**Total count "Other particles"**	**Total count "Fishbones / scales"**	***d***	**Estimated value of fish const.**
1	C	10	0,196	0,384	524	62	0,11	2,074%
2	A	10	0,185	0,326	554	21	0,04	0,676%
3	B	10	0,175	0,312	509	31	0,06	1,005%
4	B	10	0,191	0,361	542	62	0,1	1,961%
5	D	10	0,195	0,383	536	104	0,16	3,169%
6	E	10	0,071	0,108	309	168	0,35	2,501%
7	C	10	0,168	0,35	585	86	0,13	2,153%
8	E	10	0,073	0,116	482	197	0,29	2,118%
9	D	10	0,193	0,331	597	106	0,15	2,910%
10	A	10	0,176	0,323	594	10	0,02	0,291%
	Total time for the analysis of the 10 samples :	**7**						

	Laboratory identification code :	34						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	D	10,01	0,209	0,213	271	120	0,31	6,408%
2	D	10	0,232	0,237	351	59	0,14	3,339%
3	E	10,02	0,095	0,1	295	33	0,1	0,954%
4	B	10,01	0,239	0,245	375	18	0,05	1,094%
5	C	10,03	0,209	0,214	345	41	0,11	2,213%
6	E	10,05	0,097	0,103	286	104	0,27	2,574%
7	C	10,03	0,217	0,221	328	46	0,12	2,661%
8	B	10,03	0,205	0,211	394	41	0,09	1,989%
9	A	10,03	0,208	0,211	421	23	0,05	1,077%
10	A	10,03	0,187	0,191	429	4	0,01	0,173%
	Total time for the analysis of the 10 samples :	3						

	Laboratory identification code :	35						
	Sample N°	*W* (g)	*S2* (g)	*S1* (g)	Total count "Other particles"	Total count "Fishbones / scales"	*d*	Estimated value of fish const.
1	D	10	0,196	0,339	253	57	0,18	3,604%
2	A	10	0,211	0,344	522	14	0,03	0,551%
3	E	10	0,075	0,13	482	102	0,17	1,310%
4	C	10	0,184	0,312	618	109	0,15	2,759%
5	B	10	0,182	0,295	703	57	0,08	1,365%
6	D	10	0,271	0,371	539	71	0,12	3,154%
7	B	10	0,181	0,311	421	124	0,23	4,118%
8	E	10	0,085	0,139	414	308	0,43	3,626%
9	A	10	0,21	0,353	828	50	0,06	1,196%
10	C	10	0,183	0,314	524	62	0,11	1,936%
	Total time for the analysis of the 10 samples :	10						