



EURL-AP Standard Operating Procedure

On the use of the observation flowchart for light microscopy

Experts for edition and revision	
Version 1.0	Last major revision
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1. SCOPE AND PURPOSE

The purpose of the SOP is to explain, by means of examples, how to use the observation flowchart for microscopic detection of PAPs in feed. **This SOP is a binding complement to point 2.1.4.2. of Annex VI to Commission Regulation (EC) No 152/2009 as last amended by Commission Implementing Regulation (EU) No 2022/893.**

2. SUMMARY

This SOP informs the analyst how to interpret correctly point 2.1.4.2. and how to follow the observation flowchart before delivering a result by light microscopy on the presence of PAPs.

3. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS

NA

4. DEFINITIONS

Abbreviations used :

- SOP : standard operating procedure
- NA : not applicable
- PAP : processed animal proteins

5. HEALTH AND SAFETY WARNINGS

NA

6. EQUIPMENT AND MATERIALS

NA

7. STEP BY STEP PROCEDURE

7.1. Sample preparation

NA

7.2. Analysis

The observation flowchart is to be used for the disclosure of animal particles. Animal particles are categorised according to their nature: terrestrial vertebrates, terrestrial invertebrates and fish.

Observation flowchart by light microscopy

It refers to the fact that a particle can be identified as from terrestrial vertebrate origin (e.g. hairs, feathers, hydrolysed feathers, bone fragments...), from terrestrial invertebrate origin (e.g. cuticle fragments, tracheal structures of insects...), or from fish origin (e.g. scales, fishbones, otoliths, gills...).

In the observation flowcharts provided in paragraph 2.1.4.2, when in a decision diamond it is stated:

- “> 5 animal particles of each nature detected”, it means that more than 5 particles from terrestrial vertebrates **and** more than 5 particles from fish have been detected.
- “animal particles of a given nature detected”, it means that at least 1 particle from terrestrial vertebrates **or** 1 particle from fish has been detected.

For the detection of terrestrial vertebrates and fish, the detection is based on the observation of a maximum of 6 microscopic slides per determination. The same slides sets shall be used for the detection of both natures (terrestrial vertebrates and fish).

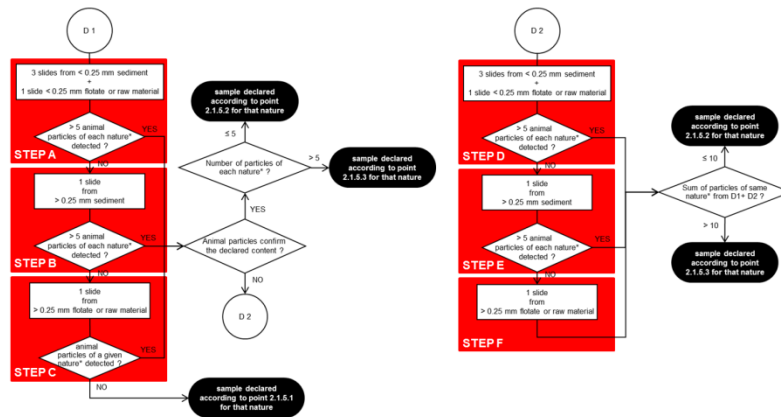
For the detection of terrestrial invertebrates, the detection is based on the observation of a maximum of 4 microscopic slides per determination, all of them prepared from the final flotote.

Results are reported in three ways: negative, positive and below the decision limit. This decision limit is a fixed threshold of a minimum of 5 particles of a given nature to observe per determination before allowing to declare a sample as truly positive. Effectively, the validation of the method demonstrated that below this level the risk of false positive result from lab cross-contamination cannot be discriminated from a truly low level presence of contamination. Since this low number of particles ensures a concentration of PAP lower than 0.1% w/w, which is considered as the required limit of detection, the sample can be considered as negative in term of risk management.

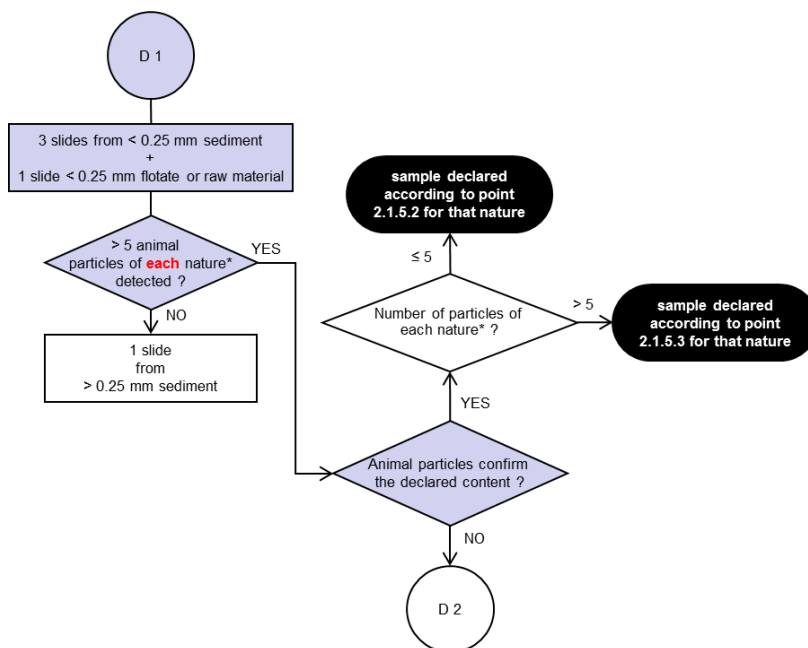
7.2.1. TERRESTRIAL VERTEBRATES AND FISH

No more than two determinations (D1, D2) shall be performed.

Some practical cases are detailed to explain the use of the flowchart and be sure of taking the right decision at each step (red squares in the figure below) of determination 1 (steps A, B and C) and of determination 2 (steps D, E and F):



Step A



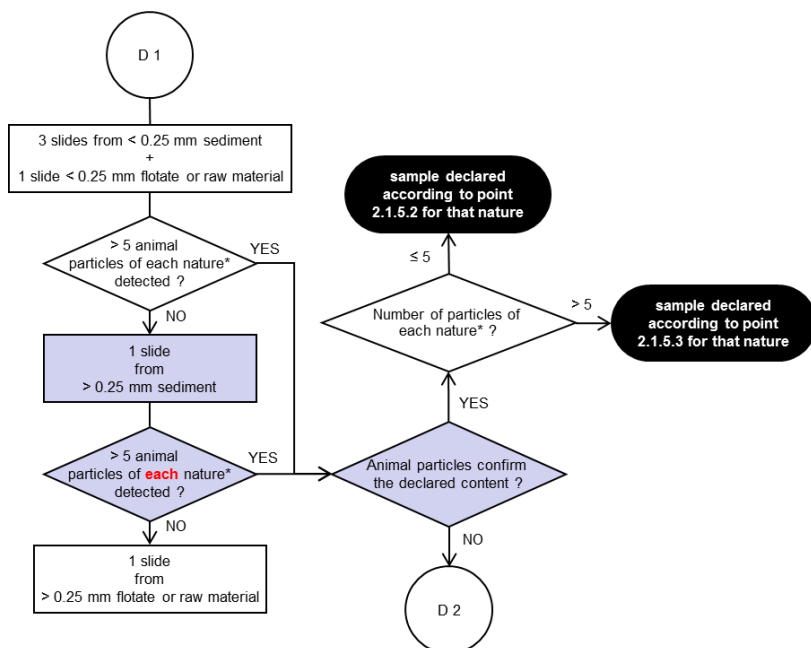
D1				
	# Terr. Ver.	# Fish		
Case 1	0	0	Continue D1	
Case 2	1	0	Continue D1	
Case 3	7	0	Continue D1	
Case 4	11	20	CONFIRMATION OF CONTENT ?	
			Y	2.1.5.3 (positive for both terr. vertebrates and fish)
			N	D2 → Case 4'
Case 5	8	5	Continue D1	

Case 3 : There are more than 5 terrestrial vertebrate particles, but no fish particles. The absence of fish is leading the decision to continue the observations until the whole sequence of D1 is realised.

Case 4 : There are more than 5 terrestrial vertebrate particles AND more than 5 fish particles. If this presence is confirming the declared content no other observation is needed and the sample is declared by using the terminology laid down in point 2.1.5.3 as positive for both natures. If this presence is not confirming the declared content the decision is to go directly to a second determination (see Step D).

Case 5 : Since no more than 5 fish particles are detected the decision is to continue the observations of the first determination

Step B

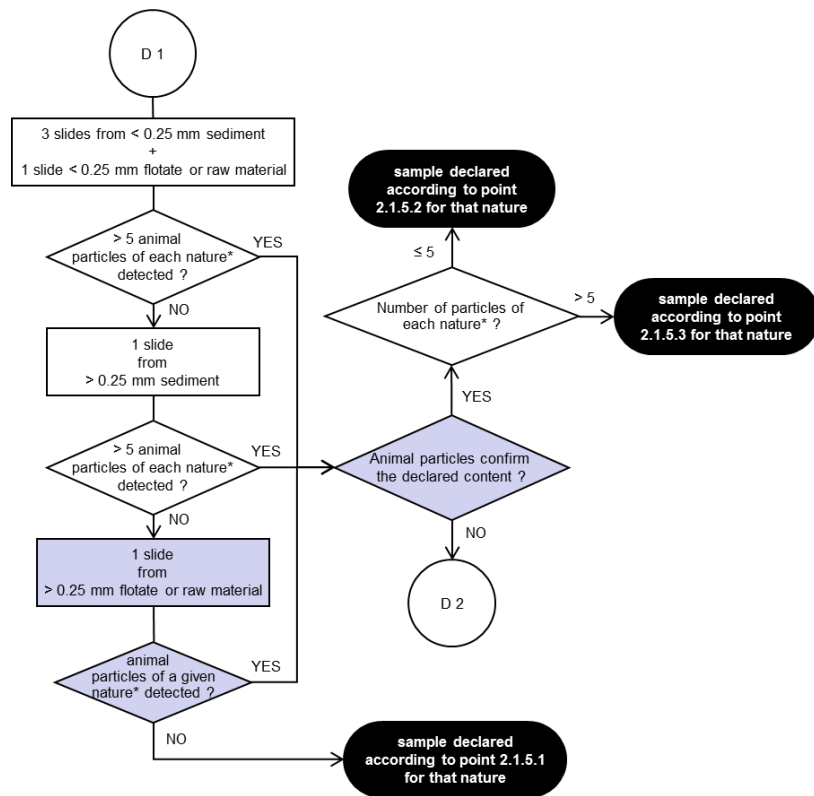


D1				
	# Terr. Ver.	# Fish		
Case 1	0	0	Continue D1	
Case 2	1	0	Continue D1	
Case 3	12	5	Continue D1	
Case 5	12	6	CONFIRMATION OF CONTENT ?	
			Y	2.1.5.3 (positive for both terr. vertebrates and fish)
			N	D2 → Case 5'

Case 3 : 5 more terrestrial vertebrate particles and 5 more fish particles were observed. The total is thus of respectively 12 and 5. Since still no more than 5 fish particles have been detected, the decision is to continue the observations of the first determination.

Case 5 : Additional terrestrial vertebrate particles and fish particles resulted in a respective total of 12 and 6 particles. If this presence is confirming the declared content no other observation is needed and the sample is declared using the terminology laid down in point 2.1.5.3 as positive for both natures. If this presence is not confirming the declared content the decision is to go directly to a second determination (see Step D).

Step C



D1				
	# Terr. Ver.	# Fish		
Case 1	0	0	2.1.5.1. (negative for both terr. vertebrates and fish)	
Case 2	1	0	CONFIRMATION OF CONTENT ?	
			Y	2.1.5.2 (< decision limit for terr. vertebrates) 2.1.5.1. (negative for fish)
Case 3	13	5	CONFIRMATION OF CONTENT ?	
			Y	2.1.5.3 (positive for terr. vertebrates) 2.1.5.2 (< decision limit for fish)
			N	D2 → Case 3'

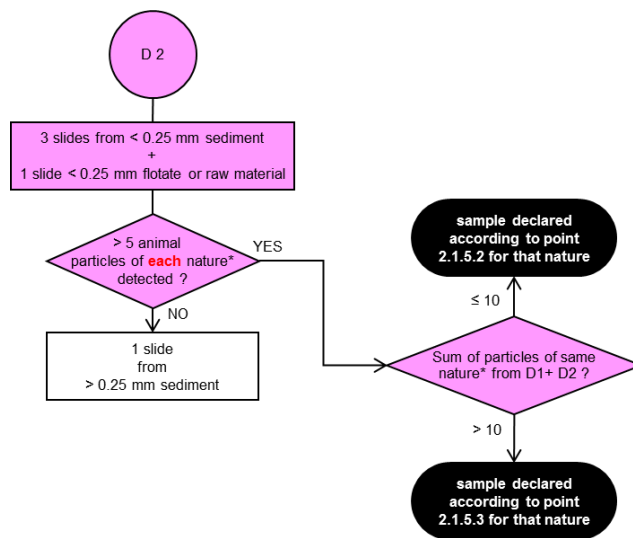
Case 1 : No animal particles were detected over the 6 slides prepared. The result has to be expressed by using the terminology laid down in point 2.1.5.1. per nature ; thus negative for terrestrial vertebrates and for fish.

Case 2 : No other animal particle was detected. If this presence is confirming the declared content no other observation is needed and the sample is declared by using terminology laid down in point 2.1.5.2 as below the decision limit for terrestrial vertebrates and according to point 2.1.5.1 as negative for fish. If this presence of a single terrestrial vertebrate particle is not confirming the declared content a second determination is required (see Step D) as imposed by point 2.1.4.3.

Case 3 : One additional terrestrial vertebrate particle resulted in a total of 13 terrestrial vertebrate particles and 5 fish particles. If this presence is confirming the declared content no other observation is needed and the sample is declared by using terminology laid down in point 2.1.5.3 as positive for terrestrial vertebrates and according to point 2.1.5.2 as below the decision limit for fish. If this presence is not confirming the declared content a second determination is required (see Step D).

Step D

The second determination is performed on a different sub-sample of 50 g which shall be ground.



	from D1		D2		
	# Terr. Ver.	# Fish	# Terr. Ver.	# Fish	
Case 2'	1	0	0	0	Continue D2
Case 3'	13	5	11	3	Continue D2
Case 4'	11	20	7	24	SUM
Case 5'	12	6	8	9	SUM

Σ	# Terr. Ver.	# Fish	
Case 4'	11 + 7	20 + 24	2.1.5.3 (positive for both terr. vertebrates and fish)
Case 5'	12 + 8	6 + 9	2.1.5.3 (positive for both terr. vertebrates and fish)

Case 3' : Several terrestrial vertebrate and fish particles were detected from the first series of slides of the second determination, 11 and 3 respectively. Continuation of the second determination is required since no more than 5 fish particles are detected.

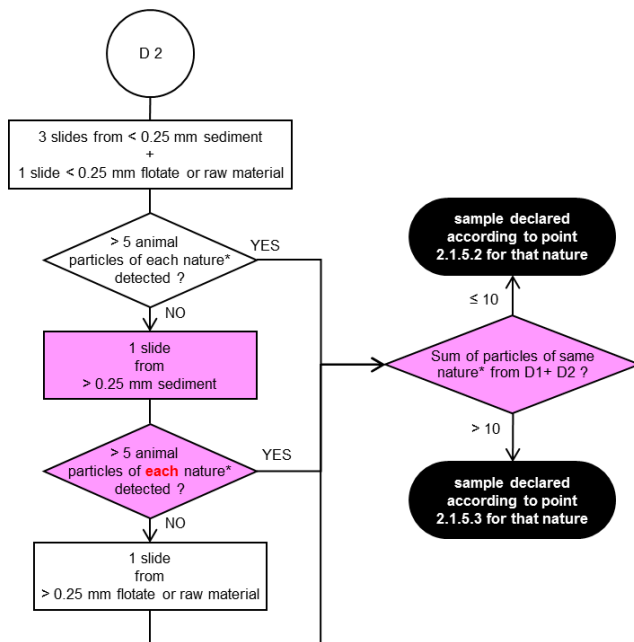
Case 4' : More than 5 particles from both terrestrial vertebrates and fish were observed. According to the flowchart the sum of all particles observed during the first and second determination has to be calculated per

Observation flowchart by light microscopy

nature. A total of 18 terrestrial particles and 44 fish particles, i.e. for both above 10 particles, allows to declare the sample by using terminology laid down in point 2.1.5.3 as positive for both natures.

Case 5' : Similarly to case 4' calculation of the sum allows to declare the sample by using terminology laid down in point 2.1.5.3 as positive for both natures.

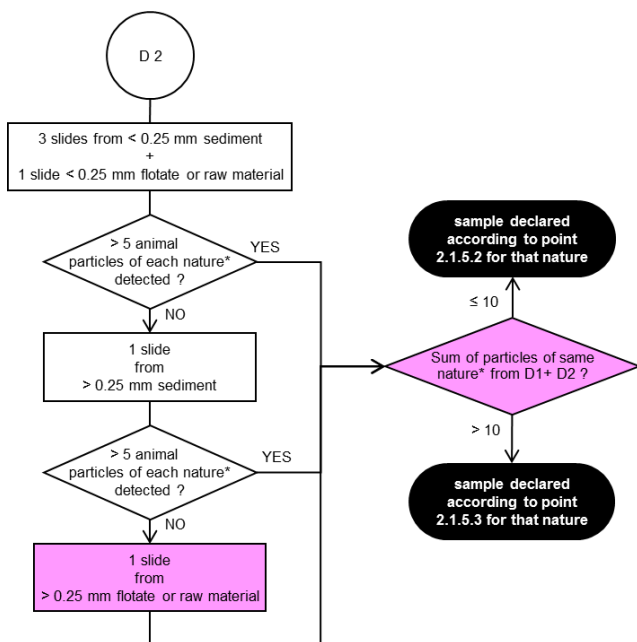
Step E



	from D1		D2		
	# Terr. Ver.	# Fish	# Terr. Ver.	# Fish	
Case 2'	1	0	0	0	Continue D2
Case 3'	13	5	15	5	Continue D2

Case 2': 4 more terrestrial vertebrate particles were detected and 2 additional fish particles. Since the number of fish particles does not exceed 5 for the second determination, D2 shall be continued.

Step F



	from D1		D2		
	# Terr. Ver.	# Fish	# Terr. Ver.	# Fish	
Case 2'	1	0	0	0	SUM
Case 3'	13	5	15	5	SUM

Σ	# Terr. Ver.	# Fish	
Case 2'	1 + 0	0 + 0	2.1.5.2 (< decision limit for terr. vertebrates) 2.1.5.1. (negative for fish)
Case 3'	13 + 15	5 + 5	2.1.5.3 (positive for terr. vertebrates) 2.1.5.2 (< decision limit for fish)

Step F constitutes the last slide observation leading to the final sum calculation.

Case 2' : Due to the presence of the single terrestrial vertebrate particle the sample has to be declared according to point 2.1.5.2 as below the decision limit of the microscopic method for the presence of terrestrial vertebrates, and according to point 2.1.5.1 as negative for fish.

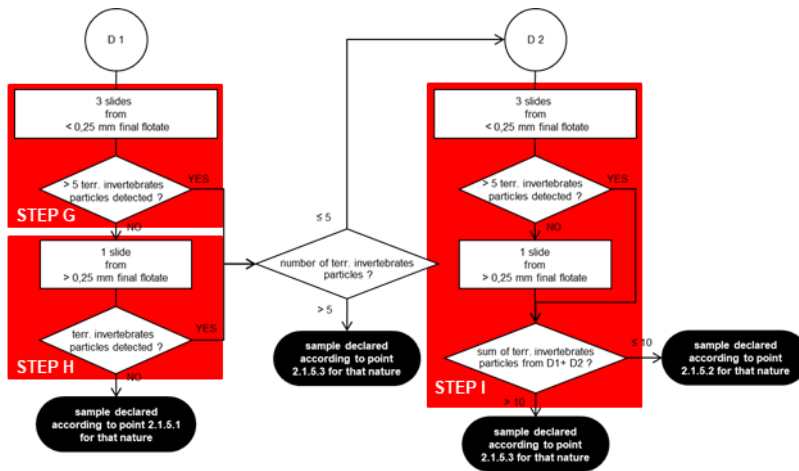
Case 3' : The sum of terrestrial vertebrate particles from the first and second determinations allow to declare the sample as positive for terrestrial vertebrates. For the presence of fish, since only 10 fish particles were identified over the two determination the sample must be declared as below the decision limit of the microscopic method for the presence of fish.

7.2.2. TERRESTRIAL INVERTEBRATES

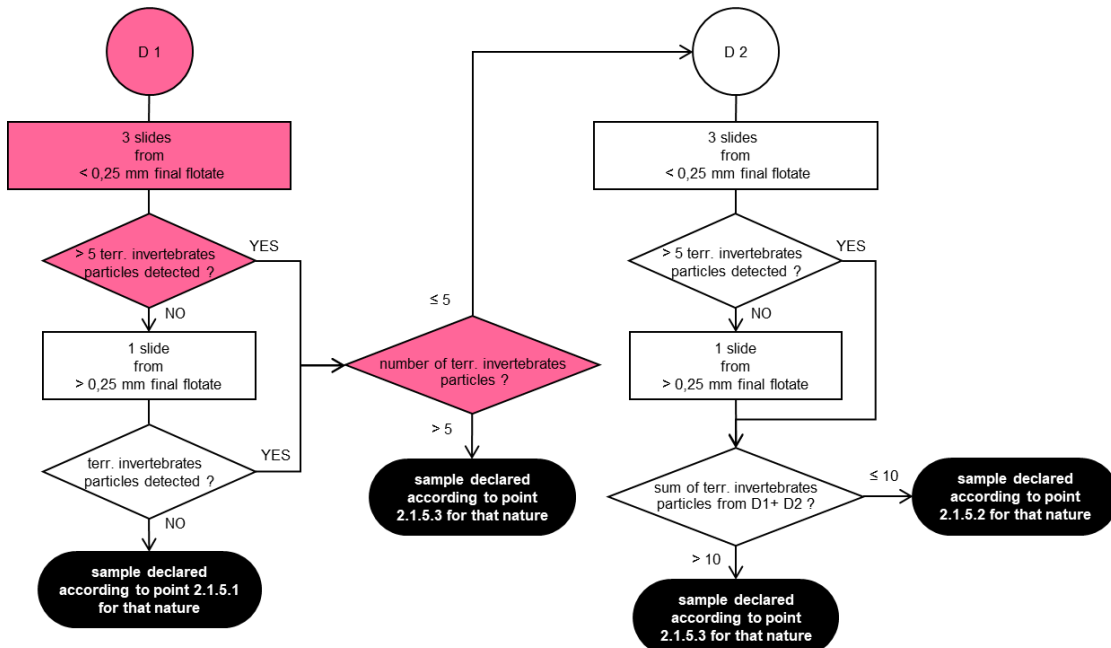
For reminder, detection of terrestrial invertebrates PAPs has only to be performed on ruminant feed or feed ingredients intended to ruminants.

No more than two determinations (D1, D2) shall be performed.

Some practical cases are detailed to explain the use of the flowchart and be sure of taking the right decision at each step (red squares in the figure below) of determination 1 (steps G and H) and of determination 2 (step I):



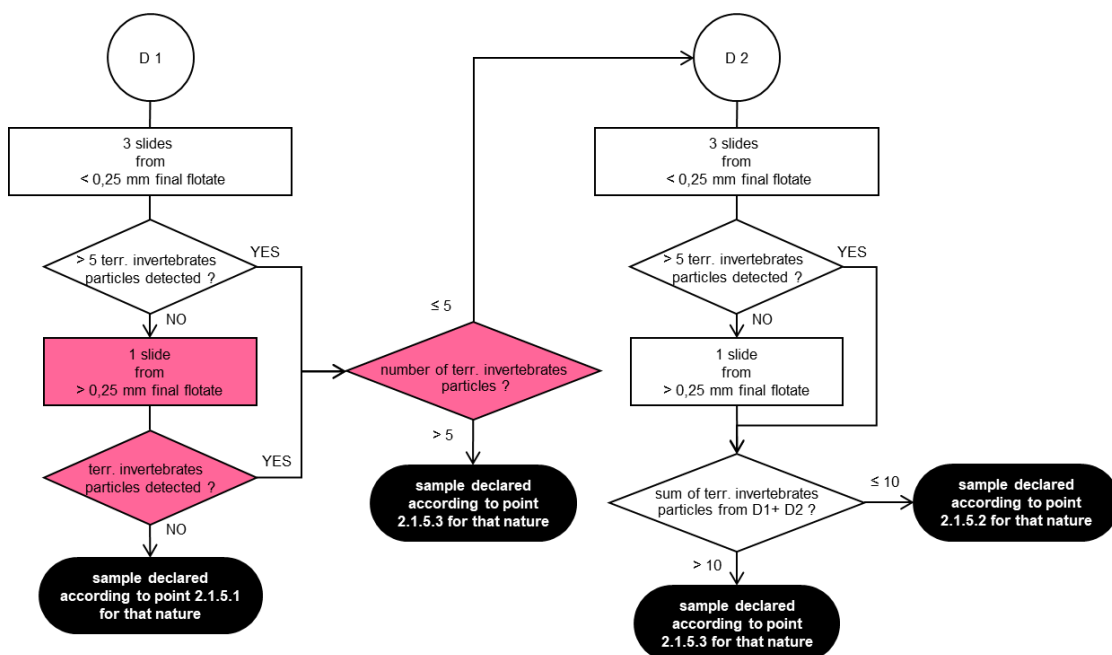
Step G



D1		
	# Terr. Inver.	
Case 6	0	Continue D1
Case 7	1	Continue D1
Case 8	2	Continue D1
Case 9	7	2.1.5.3 (positive for terr. invertebrates)

Case 9 : Seven particles from terrestrial invertebrates were detected. There is no need to observe additional slides. The result has to be expressed by using the terminology laid down in point 2.1.5.3. thus positive for terrestrial invertebrates.

Step H



D1		
	# Terr. Inver.	
Case 6	0	2.1.5.1. (negative for terr. invertebrates)
Case 7	3	D2
Case 8	11	2.1.5.3 (positive for terr. invertebrates)

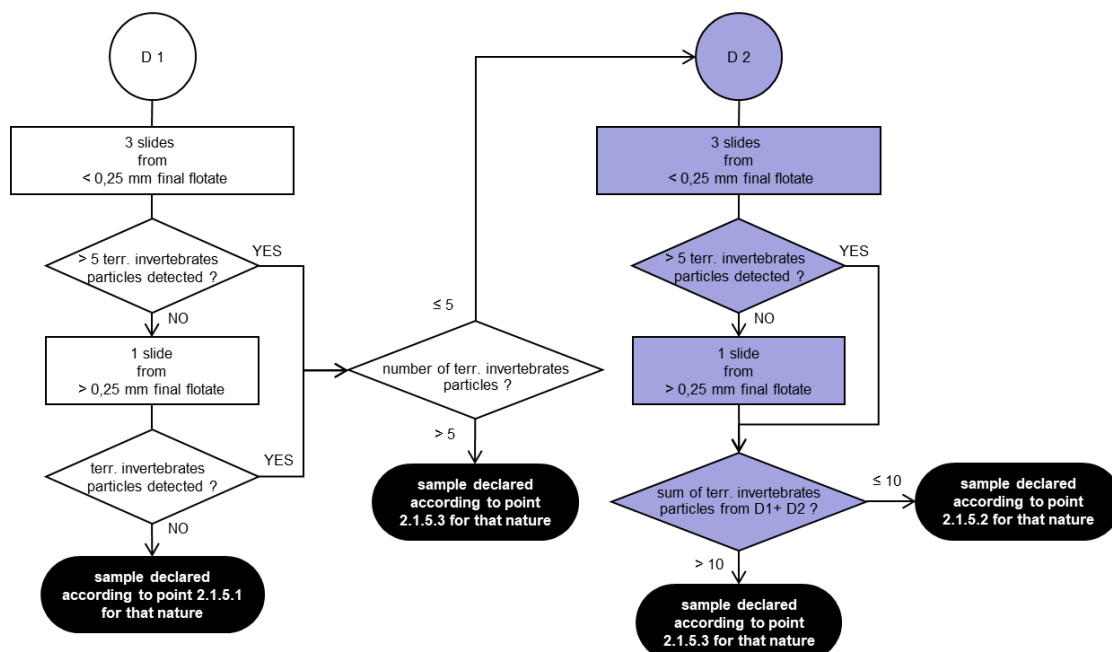
Observation flowchart by light microscopy

Case 6 : No particles from terrestrial invertebrates were detected over the 4 slides prepared. The result has to be expressed by using the terminology laid down in point 2.1.5.1. thus negative for terrestrial invertebrates.

Case 7 : 2 more terrestrial invertebrate particles were detected. Since the number of terrestrial invertebrate particles does not exceed 5, a second determination needs to be performed.

Case 8 : 9 more particles from terrestrial invertebrates were detected resulting in a total of 11 over the 4 slides prepared. The result has to be expressed by using the terminology laid down in point 2.1.5.3. thus positive for terrestrial invertebrates.

Step I



	from D1	D2	
	# Terr. Inver.	# Terr. Inver.	
Case 7	3	#	SUM

Σ	# Terr. Inver.		
Case 7	3 + #	if SUM > 10	2.1.5.3 (positive for terr. invertebrates)
		if SUM ≤ 10	2.1.5.2 (< decision limit for terr. invertebrates)

Case 7 : During the second determination, # additional particles from terrestrial invertebrates were detected. When summed to the 3 particles obtained during D1 two options may originate. If the total number of terrestrial invertebrate particles from D1 and D2 is exceeding 10, then the sample is declared by using the terminology laid down in point 2.1.5.3. thus positive for terrestrial invertebrates. If this total is inferior of equal to 10, then the sample is declared accordingly to point 2.1.5.2. thus below the limit of decision of the microscopic method for the presence of terrestrial invertebrates.

8. INTERPRETATION OF RESULTS

Some microscopically observable particles cannot be credited to one or the other category (e.g. muscle fibres, cartilage not bond to a bony area). These particles can only be described as from animal origin. For the interpretation and expression of the result:

- When only such particles (e.g. muscles) are found (i.e. no particle credited to either terrestrial vertebrates, terrestrial invertebrates or fish) the report shall mention that e.g. only muscle fibres were detected and that it cannot be excluded that they originate from terrestrial vertebrate origin.
- When such particles (e.g. muscles) are found in combination with (A) only particles of terrestrial vertebrate origin (e.g. bones) or (B) only particles of terrestrial invertebrates (e.g. cuticular fragments) or (C) only particles of fish origin (e.g. fishbones) they shall be classified accordingly as (A) terrestrial vertebrates or (B) terrestrial invertebrates or (C) fish.
- When for instance such particles (e.g. muscles) are found in combination with particles of both terrestrial vertebrates and fish, they shall be classified as terrestrial vertebrates and fish according to the same ratio of terrestrial vertebrates and fish identified structures. Example: a total of 12 muscle fibres have been detected in presence of 2 terrestrial bones and 6 fishbones. The ratio of terrestrial vertebrates particles is $2/(2+6) = 1/4$. Therefore, 3 muscle fibres shall be classified as terrestrial vertebrates and 9 as fish.

Blood particles shall be considered as from terrestrial vertebrate origin since, as far as it is known, no such products are processed from fish. When such particles are found in fishmeal, it is reasonable to consider them as from fish origin after having checked the absence of DNA from terrestrial vertebrates (ruminant, porcine and poultry) by validated PCR methods.

9. REFERENCES

Commission Implementing Regulation (EU) 2022/893 of 7 June 2022 amending Annex VI to Regulation (EC) No 152/2009 as regards the methods of analysis for the detection of constituents of terrestrial invertebrates for the official control of feed. Official Journal of the European Union, L155, 8.6.2022, 24-35

Version	Publication date	Application date	Repeal date
2.0	26.10.2022	31.10.2022	
1.0	26.11.2020	26.12.2020	26.10.2022