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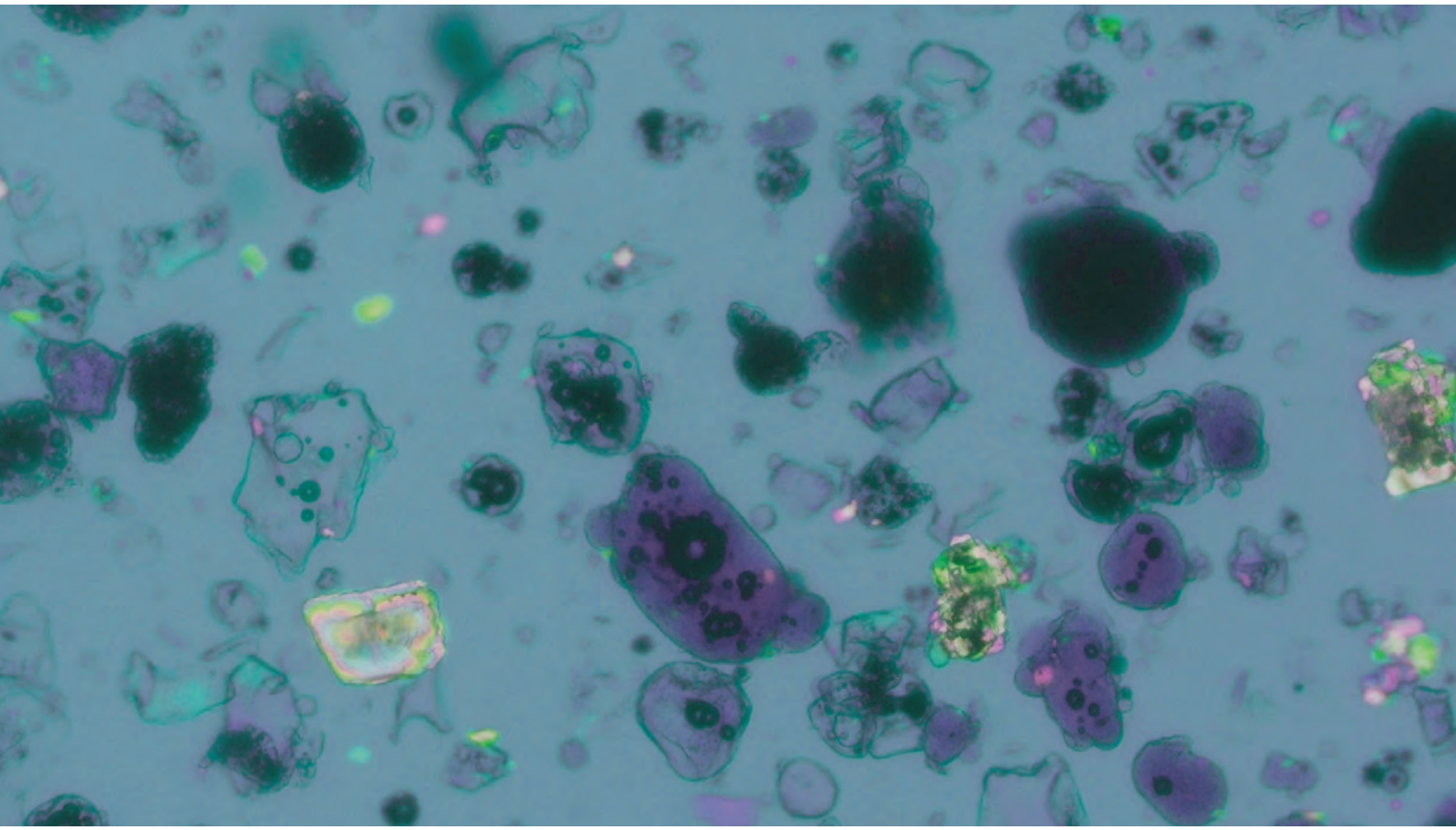

Investigation into blood plasma in milk formula

RIKILT Report 2011.003

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Report 2011.003 **October 2011**

Project number: 120.72.357.01
BAS-code: WOT 02-004-001
Project title: NRL animal proteins

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This investigation was funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation and was carried out within the WOT Programme on Food Safety.

Distribution list:

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Summary

In July 2010 a Rapid Alert was issued by Spain concerning the presence of blood plasma in milk formula ("KUVO") for calves. The blood plasma was detected by a microscopic staining method using tetramethylbenzidine (TMB).

RIKILT investigated the suspect research sample by applying the standard research method for animal proteins and several additional staining methods, including TMB (macroscopic and microscopic). Without any further information on the procedure in Spain, the TMB method was applied according to the standard protocol. A slight green response was found in the suspect research sample after TMB staining, which could have indicated the presence of blood plasma. As a comparison, other products such as skimmed milk powder, six milk formulas, blood meal and blood plasma were investigated using the TMB method (Table 3). Blood meal and blood plasma showed a strong colouring response, while the blank skimmed milk powder did not show colouring within the fixed reaction period of the method. Four out of six KUVOs showed a slight colour response to the TMB staining (Figure 5), comparable to that of the suspect sample. The blank KUVO with the lowest response was used to make a series of samples with artificial contamination with blood plasma between 0.05% and 1%. A colouring response was found at levels of 0.5% and higher.

The method used with TMB is a screening method which has to be followed by a confirmation method for the presence of animal proteins in the applied form (here: blood plasma). However, as far as known, such confirmation methods have not been applied in Spain, nor are they operational for animal proteins at RIKILT.

In addition, real-time PCR was applied, because a German laboratory reported porcine DNA in the suspect batch. These results were not confirmed (Table 5), which indicates a level of contamination lower than 1.0%, if any. Bovine DNA was detected in all samples, which is an expected result from milk powders or milk feeds. The extracted DNA proved to be of low quality.

It can be concluded that the presence of porcine blood plasma in samples originating from the suspected batch could not be confirmed by RIKILT. The accuracy of the results is validated by the investigation of a large series of positive and negative test samples. It should be noted that the TMB colouring method as used in Spain as well as by RIKILT is currently neither applicable nor validated for the detection of blood material in a matrix of milk powder. The presence of blood plasma in the suspect research sample was not confirmed reliably with any of the applied research methods.

Contents

1 Introduction

In June 2010 Spain issued a Rapid Alert concerning the presence of blood plasma in milk formula for calves (RASFF information, 11 June 2010). The notification stated that a sample of milk formula had tested positive on the basis of a microscopic method with no further specification (reference unknown). The finding was subsequently confirmed with tetramethylbenzidine (TMB) staining. TMB stains blood and blood products bright green (Garner et al., 1976). The owner of the consignment ordered an additional investigation at a German laboratory, which performed a PCR test and found a low level of porcine DNA.

TMB staining is based on endogenous peroxidase haemoglobin activity in blood. It is used primarily in forensic diagnostics; the sensitivity for blood is set at 1 ppm (Garner et al., 1976). It is a screening method that can produce false positive results when other substances, including some metals and phytochemicals (such as garlic), are present.

The authorised institute in the Netherlands, the new Food and Consumer Product Safety Authority (Voedsel en Waren Autoriteit, VWA), asked RIKILT to analyse the sample from the suspect consignment by applying all relevant additional tests that are normally carried out in the Netherlands. It also asked RIKILT to evaluate the procedure applied in Spain on the basis of the assumption that the TMB staining was performed according to the standard protocol, as there was no further information to this effect in the Spanish documentation or in later correspondence.

2 Materials and methods

RIKILT analysed the suspect samples and various control samples by applying the standard method for the microscopic examination of animal proteins (Regulation (EC) 152/2009). Various staining techniques were also applied: a few standard tests for milk powder, TMB and DAB+ staining for endogenous peroxidase activity, standard histological HE staining, and real-time PCR for species identification.

Six milk formulas (KUVOs) were analysed to find a suitable blank reference. A sample of garlic powder was added to this series as a positive control. In order to estimate the amount of any blood plasma, a serial dilution was made of the blank KUVO from the series with the lowest background colouration, which was artificially contaminated with blood plasma at four levels.

2.1 Materials

Two samples of the suspect consignment of milk formula were obtained via the VWA. A reference sample of porcine blood plasma was requested from Sonac.

The following materials were used:

- Two samples of milk formula from the suspect consignment:
  - VWA no. 66376923 (RIK no. 251359) for PCR testing;
  - VWA no. 66376931 (RIK no. 251360) for microscopic examination.
- Blank milk formulas (KUVOs) (n = 6)
- Blank skimmed milk powder (MMP)
- Blood meal (BM)
- Garlic powder
- (Porcine) blood plasma powder p70, (Sonac 2010) (BPp70)
- Blank milk formula (KUVO) to which 0.05% BPp70 was added
- Blank milk formula (KUVO) to which 0.1% BPp70 was added
- Blank milk formula (KUVO) to which 0.5% BPp70 was added
- Blank milk formula (KUVO) to which 1.0% BPp70 was added

The last four samples were obtained by adding 0.05, 0.1, 0.5 and 1 gram of blood plasma powder to 100 grams of blank milk formula and mixing it well.

2.2 Methods

The following methods were used:

2.2.1 Composition and authenticity of milk powder and milk formulas

Observations were carried out on the basis of various microscopic preparations to determine the identity of the sample. An expert system was used for this (van Raamsdonk, 2001).

2.2.2 The European harmonised microscopy method for the detection of animal proteins in animal feed and feed ingredients

This method is described in Regulation (EC) 152/2009. It is used to detect animal meal in feed by means of various markers, such as animal and fish bone fragments, hair, feather filaments, and scales (Gizzi et al., 2003; van Raamsdonk et al., 2011). Animal feed is regarded as a vegetable matrix, so the detection of blood plasma in an animal matrix (milk powder) is beyond the scope of this method. It was applied without the sedimentation step. It involves the preparation of several samples, which are then examined microscopically at different magnitudes to ascertain animal proteins (without staining).

2.2.3 Macroscopic and microscopic examination with TMB staining

This method detects blood. It is based on the peroxidase activity of haem and is used primarily in forensic research.

Chemicals: 30% hydrogen peroxide, glacial acetic acid, tetramethylbenzidine (TMB).

Two solutions were made:

- A TMB solution consisting of 0.2 grams of TMB and 10 ml of glacial acetic acid were mixed in a 50 ml beaker until the TMB had completely dissolved. This solution can be kept in a refrigerator for a week.
- A 3% hydrogen peroxide solution in which 90 ml of purified water was added to 10 ml of 30% hydrogen peroxide. This solution can be kept for a year.

The material was smeared on a slide and three drops of the TMB reagent were applied. A blue-green reaction indicates the presence of a chemical oxidant, which means that the test is unreliable. If no reaction was observed, three drops of 3% peroxidase solution were applied and the material was observed macroscopically and microscopically for blue-green colouration. A reaction indicates peroxidase activity and a positive result. No reaction indicates either that there are no blood products present or that the content of any blood products is below the detection level of the test.

Observation should start within a few minutes of applying the second solution. After 20 minutes, false positive staining may occur, which has nothing to do with peroxidase activity in the blood products. All observations were performed within the fixed period on the basis of the samples themselves. The photos serve only as illustration.

2.2.4 Microscopic examination with DAB staining

This method is used in immunohistochemistry to stain labelled antigens. The principle is based on the peroxidation of a dye.

DAB+ (DakoCytomation, Heverlee, Belgium) consists of two solutions:

- DAB+ chromogene: a solution of 3.3 diaminobenzidine (the concentration was not specified by producer).
- DAB+ substrate buffer: imidazole-HCL buffer, pH 7.5 with hydrogen peroxide and an antimicrobial agent.

Method: add 1 drop of DAB+ chromogene to 1 ml of DAB+ substrate buffer, mix well to make the DAB+ solution. This solution can be kept for one day.

The sample was smeared on a slide and a few drops of the DAB+ solution were applied.

The reaction should occur within 5-30 minutes. Dark brown denotes a positive result based on peroxidase reaction. The samples are then assessed under a microscope.

2.2.5 PCR test

Real-time PCR testing was used to detect ruminant (Mendoza et al., 2004), bovine (Brodmann et al., 2007) and porcine (Laube et al., 2003) DNA.

It should be noted that real-time PCR testing detects the DNA of the species; it does not confirm the presence of blood (products).

3 Results

3.1 Microscopic examination

3.1.1 Composition

The sample from the suspect consignment (RIKILT no. 251360) consisted of a spray-dried mixture of constituents. The composition is shown in Table 1, and is illustrated in Figure 1.

Table 1. Composition of the research sample as deduced from microscopic examination.

Constituents:	Estimated content
Full-cream milk powder	50-60%
Buttermilk powder	10-20%
Whey powder	10-20%
Undefined irregular particles	5-10%
Lactose crystals	<5%
Modified starch, presumably wheat	<1% (traces)

The VWA requested a list of ingredients from the supplier (see Table 2).

Table 2. Supplier's list of ingredients in the sample

Product	%
Wheat proteins	7.47
Lactose	3
Whey permeate	13.5
Acid whey powder	12
Powdered milk for infants	38
Casein	16.53
MSA whey powder	10

The presence of wheat protein (Table 2) could not be determined microscopically. The different types of whey powder (total: approx. 35%) accounted for a greater proportion of the ingredients than was found in the microscopic examination (Table 1: 10-20 %). Sprayed particles of blood plasma may to some extent explain the "undefined, irregular pellets".

3.1.2 European standard method for detecting animal proteins

The research sample and several control samples (blood meal, blood plasma, KUVOs) were examined according to the shortened EC procedure (Regulation (EC) 152/2009) for identifying animal proteins in animal feed. No animal proteins were found in any of the samples. This was officially reported as "Insofar as can be microscopically observed the sample contained no constituents from terrestrial animals." This conclusion is explainable since the method itself

indicates that a different protocol (unspecified) is required for the detection of blood plasma. This picture was confirmed by the analysis of a positive control sample (KUVO contaminated with blood plasma) in which identifiable traces were ascertained with the European standard method for detecting animal proteins.

3.2 Staining methods

The samples were tested with TMB, DAB+ and HE staining. Macroscopic and microscopic observations were performed. The results are shown in Table 3 and in Figures 2, 3 and 4. A negative result means that no reaction was observed. A weak positive result means that light green colouration was observed or that the sample was stained in a few places. A positive result means that the whole sample turned green.

Table 3. Results of TMB and DAB+ staining. Positive results for the control samples are shown in green, false positive or false negative results are shown in red. The results for the research sample are shown in yellow. Meaning of the signs: -: no staining, +:weak staining, ++: staining, +++: strong staining, < + >: local staining only.

Sample	Result		
	TMB macroscopic	TMB microscopic	DAB+ microscopic
251360 (VWA)	+	< + >	< + >
pos. control: blood plasma (BPp70)	+++	+++	-
pos. control: blood meal (BM)	+++	+++	-
neg. control: MMP	-	N.A.	N.A.
neg. control: KUVO 953	+	+	N.A.
neg. control: KUVO 514	+	+	N.A.
neg. control: KUVO 681	+	+	N.A.
neg. control: KUVO 062	+	+	N.A.
neg. control: KUVO 846	-	-	N.A.
neg. control: KUVO 360	-	-	N.A.
pos. control: KUVO with 0.05% blood plasma	-	-	N.A.
pos. control: KUVO with 0.1% blood plasma	-	-	N.A.
pos. control: KUVO with 0.5 % blood plasma	+	+	N.A.
pos. control: KUVO with 1.0% blood plasma	+	+	N.A.
Garlic powder	++	++	N.A.

The results in Table 3 and Figures 3 and 4 show strong staining for blood meal and blood plasma and light and local staining for the suspect sample, which is indistinguishable from the negative control. Under microscopic examination the (undissolved) plasma particles that contaminated a blood-plasma-enriched KUVO were bright green (Figure 2, left), and local green staining was observed in the suspect sample (Figure 2, right). The colouration could not be localised; it was diffuse, as if the material had dissolved.

A blank KUVO also showed green colouration, whereas the blank skimmed milk powder showed no colouration at all. This finding was followed up with an examination of a series of six blank KUVOs.

Since the literature said that garlic would give false positive results, a garlic sample was included. A weak positive result was found for four of the six KUVO samples (Figure 5). The garlic sample was completely green (Figure 5).

The sample with the least background staining (sample 846) was used for the serial dilution. The KUVOs enriched with blood plasma showed slight green staining (Figure 4), starting from enrichment of 0.5%.

The DAB+ procedure, which was tested as a possible alternative to TMB, resulted in strong colouration in certain particles in the VWA sample, but blood meal and blood plasma did not dissolve in the DAB+ or stain. Accordingly, there were no positive controls and this procedure cannot supplement the TMB procedure.

TMB, DAB+ and HE are screening procedures which should be followed by confirmation. HPLC for specific animal proteins is an option but it is not implemented at RIKILT. Real-time PCR was therefore chosen for the detection of DNA.

3.3 PCR confirmation

PCR was performed to detect the presence of bovine and porcine DNA and to confirm the visual and microscopic observations. The results were mixed (Table 4).

Table 4. Results of the DNA detection with PCR. Green indicates correct results; red indicates false negative results, yellow indicates the results of the research sample. Meaning of signs: --: not detected, +: detected.

Sample		Test	
		DNA pig (PCR RIKILT)	DNA cattle (PCR RIKILT)
sample:	251359 (VWA)	--	+
pos. control:	KUVO + 0.05% plasma	--	+
pos. control:	KUVO + 0.1% plasma	+	+
pos. control:	KUVO + 0.5% plasma	--	+
pos. control:	KUVO + 1.0% plasma	+	+
neg. control:	MMP	--	+
neg. control:	KUVO	--	+
pos. control:	bovine reference	--	+
pos. control:	porcine reference	+	--
neg. control:	water	--	--

The negative controls, blank powdered milk and blank milk formula, and the positive control on the bovine and porcine reference material delivered correct results. Porcine blood plasma was found at contamination levels of 1% and 0.1% in a series of plasma-enriched milk formula but not at 0.5% or 0.05%. The suspect sample did not respond to the porcine test. A PCR test for bovine DNA did deliver a positive result, but this could be expected for DNA extraction from milk formula. The quality of the extracted DNA proved to be low.

4 Discussion

A sample from a suspect consignment of milk formula was examined after a Rapid Alert from Spain had reported the presence of blood plasma. The method that was used in Spain to detect the blood plasma – tetramethylbenzidine staining – was also tested.

Given the nature of the question and the legal principles, the method prescribed by Regulation (EC) 152/2009 for the detection of animal proteins was applied. Animal proteins were "defined as products obtained from the processing of the carcases and parts of the carcases of mammals, poultry and fish." Blood plasma is not a marker that lends itself for detection under a microscope (Gizzi et al., 2003; van Raamsdonk et al., 2011). This explains the negative findings that were ascertained with the official method.

In the RIKILT investigation TMB staining was performed on blood meal and blood plasma, which assumed a bright green colour. Skimmed milk powder tested negative but blank KUVO turned green after a while and therefore tested positive. Weak local staining was observed in the suspect sample. This was in keeping with the findings in Spain (RASFF 2010.0762-add 6).

As the TMB test was not entirely negative for the blank KUVO, six different KUVOs were tested. Only two proved negative. Green spots appeared on the other samples after a few minutes, which made them comparable with the suspect sample.

The (false) positive reaction of other substances with endogenous peroxidase activity, such as chemical oxidants, certain metals, catalysts and vegetable peroxidases (Winchester and Wansbrough, s.n.) and garlic (Garner et al., 1976) explains the green staining in some blank milk formula in this investigation. Garlic powder also tested positive. It may be concluded from these results that the applied method could not demonstrate the presence of blood plasma in the suspect sample.

In the DAB+ procedure, which was tried out as a possible alternative to TMB, strong staining was observed in certain grains in the research sample, but neither blood meal nor blood plasma dissolved in the DAB+ or assumed a colour. As a result, there were no positive controls and this method cannot a supplement TMB. The smears stained with HE showed fat particles and cell nuclei, which did not provide any additional information.

The suspect sample tested positive for pig DNA at a commercial laboratory in Germany (VWA report). The real-time PCR porcine test which RIKILT applied to the sample was negative; all controls were correct (Fryganas, 2011). These results may be attributable to the poor quality of the extracted DNA and/or a low level of contamination. The contamination level ascertained with the PCR test (Table 5) seems, on the basis of the current results, to be lower than the level ascertained with the TMB test (Table 3).

5 Conclusion

TMB is a screening procedure that detects the presence of blood products. It has not been validated in any way whatsoever for the animal feed matrix in general and milk formula in particular. TMB staining produced a weak result in the sample which reportedly contained blood plasma. Similar results were, however, found in four out of six blank KUVOs. It must be concluded therefore that the presence of blood plasma in the suspect sample could not be confirmed with microscopic examination.

PCR analyses were performed because test findings by a laboratory in Germany indicated that porcine plasma had been used. This could not be confirmed. The detection limit for PCR (approx. 0.1%) indicates that there was no contamination at a high level. However, PCR detects only (the DNA of) the species and not the presence of blood (products).

It should be noted that the total package of results obtained with TMB staining and real-time PCR detection of DNA was not optimised for the animal feed matrix in general or for the milk powder matrix in particular.

Recommendations

Recommendations for a validated method of analysis:

- Develop a procedure to confirm the presence of blood plasma in milk formula.
- Conduct further research on the possible contamination of KUVOs with blood plasma.
- Conduct further research on the value of the TMB test in KUVO analyses.

Bibliography

Brodmann, P.D. and Moor, D., 2003. Sensitive and semi-quantitative TaqMan(TM) real-time polymerase chain reaction systems for the detection of beef (*Bos taurus*) and the detection of the family Mammalia in food and feed. *Meat Science* 65(1): 599-607.

Fryganas, Chr., 2011. *Animal species detection and identification with real-time PCR. Validation of animal species specific TaqMan (r) systems.* Doctoral Thesis, Wageningen University and RIKILT Institute of Food Safety.

Garner, D.D., Cano, K.M., Peimer, R.S., and Yeshion, T.E., 1976. An Evaluation of Tetramethylbenzidine as a Presumptive Test for Blood. *Journal of Forensic Sciences* 21, No. 4.

Gizzi, G., van Raamsdonk, L.W.D., Baeten, V., Murray, I., Berben, G., Brambilla G., & von Holst, C., 2003. An overview of tests for animal tissues in animal feeds used in the public health response against BSE. In: Risk analysis of BSE and TSEs: update on BSE and use of alternatives to MBM as protein supplements. *Rev. Sci.Tech.Off. Int. Epiz.* 22(1): 311-331.

Laube, I., Zagon, J., et al., 2007. Development and design of a 'ready-to-use' reaction plate for a PCR-based simultaneous detection of animal species used in foods. *International Journal of Food Science & Technology* 42(1): 9-17.

Mendoza-Romero, L., Verkaar, E.L.C., Savelkoul, P.H., Catsburg, A, Aarts, H.J., Buntjer, J.B., Lenstra, J.A., 2004. Real-Time PCR Detection of Ruminant DNA. *Journal of Food Protection* 67: 550-554.

Van Raamsdonk, L.W.D. Decision Support System ondersteunt microscopisch onderzoek. *VMT* 2001(11): 41-43.

Van Raamsdonk, L.W.D., Pinotti, L., Veys, P., Bremer, M., Hekman, W., Kemmers, A., Campagnoli, A., Platanin, C., Belinchón Crespo, C., Vliege, J., Pinckaers, V., Jørgensen, J.S. New developments in classical microscopy; what can be expected for the official control? *Biotechnol. Agron. Soc. Environ*. 15(S1), 15-24.

RASFF information, 11 June 2010, information notification 2010.0762, original notification.

RASFF information, 17 June 2010, information notification 2010.0762-add01.

RASFF information, 20 July 2010, information notification 2010.0762-add05.

RASFF information, 4 August 2010, information notification 2010.0762-add06.

Tetramethylbenzidine presumptive test for blood. http://static.dna.gov/lab-manual/Linked%20Documents/Protocols/pdi_lab_pro_2.19.pdf.

Winchester, R.V. and Wansbrough, H. Blood detection by chemical methods. http://nzic.org.nz/ChemProcesses/biotech/12A.pdf.

Annex I
Figures

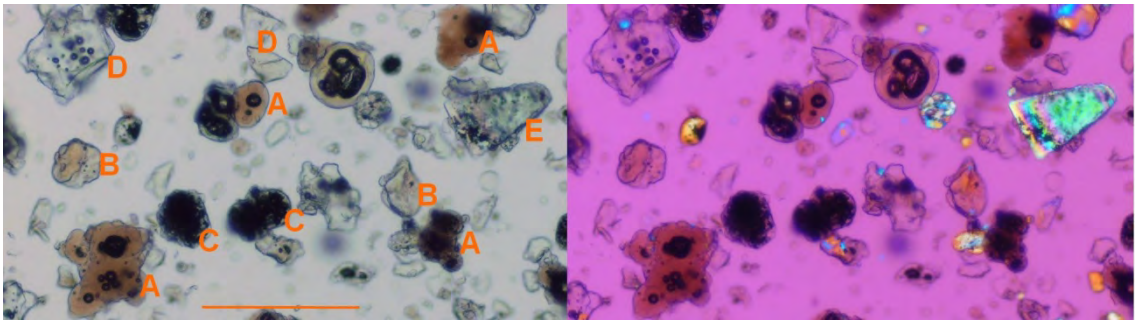


Figure 1. Research sample 251360, with particles embedded in paraffin oil, visible in normal light (left) and under polarised light (right). Bar: 100 µm. Explanation of letters: A: full-cream milk powder, B: buttermilk powder, C: skimmed milk powder with high air content, D: undefined particles, E: lactose crystal.

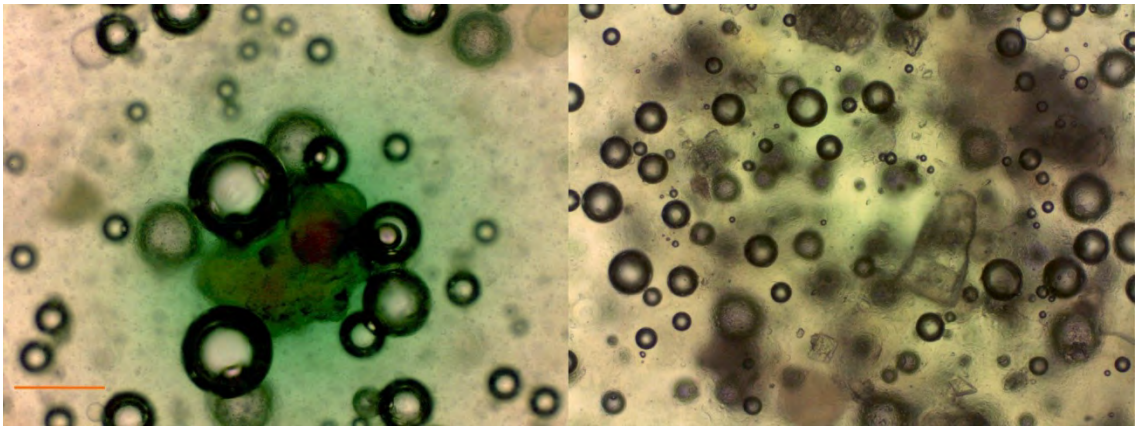
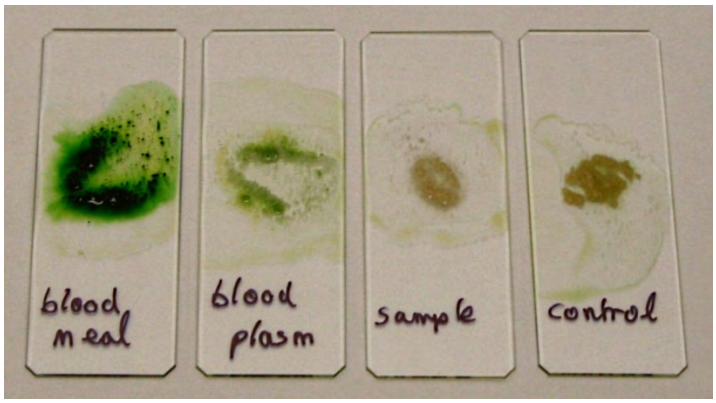


Figure 2. Colouration of a particle of blood plasma in an artificially contaminated KUVO (left), and slight colouration in the research sample (right) after TMB staining. Bar: 100 µm.



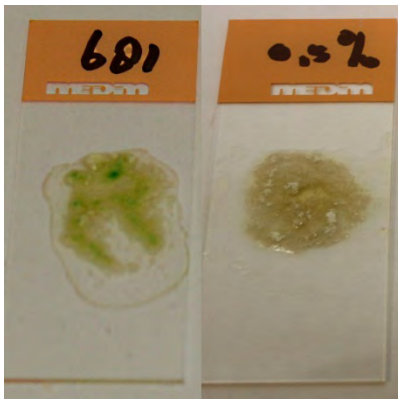


Figure 3 (left). Tetramethylbenzidine test on blood meal, blood plasma, the research sample, and a negative control (milk formula). Figure 4 (right). Staining of blank milk formula (KUVO 681) and milk formula contaminated with 0.5% blood plasma.

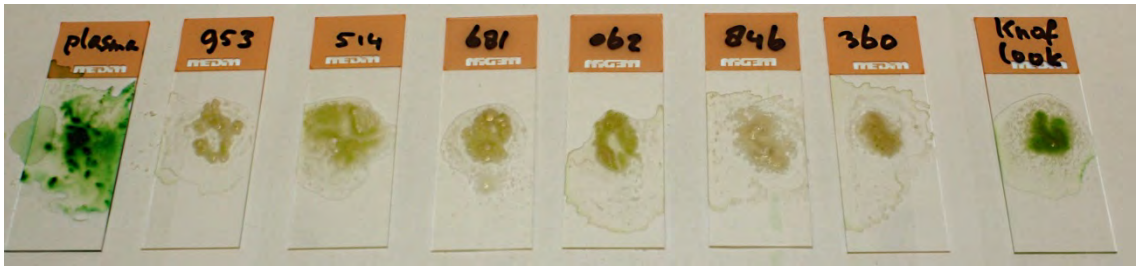


Figure 5. Colour reactions of blood plasma, six different blank milk formulas (KUVO) and garlic powder.

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RIKILT advises national and international governments on establishing standards and methods of analysis. RIKILT is available 24 hours a day and seven days a week in cases of incidents and food crises.

The research institute in Wageningen is the National Reference Laboratory (NRL) for milk, genetically modified organisms, and nearly all chemical substances, and is also the European Union Reference Laboratory (EU-RL) for substances with hormonal effects.

RIKILT is a member of various national and international expertise centres and networks. Most of our work is commissioned by the Dutch Ministry of Economic Affairs, Agriculture and Innovation and the new Dutch Food and Consumer Product Safety Authority. Other parties commissioning our work include the European Union, the European Food Safety Authority (EFSA), foreign governments, social organisations, and businesses.

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