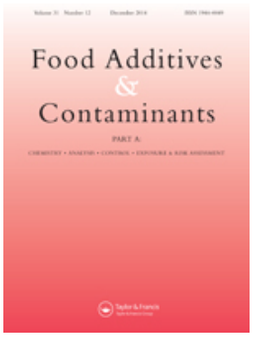

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Validation of a lateral flow test (MRLAFMQ) for the detection of aflatoxin M_1 at 50 ng l^{-1} in raw commingled milk

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Aflatoxin M_1 contamination in dairy products is a risk when feedstuff contaminated with aflatoxin B_1 produced by moulds is consumed by milk-producing animals. Milk can be screened for aflatoxin M_1 at the European Union maximum limit of 50 ng l^{-1} by a lateral flow test, the MRLAFMQ (Aflatoxin M1) Test. The method takes 15 min with no milk dilution or a sample preparation step. The lateral flow assay was validated at the Technology and Food Science Unit of the Institute for Agricultural and Fisheries Research (ILVO-T&V) according to European Union guidelines using fortified raw milk samples. A detection capability of 50 ng l^{-1} was demonstrated with a false negative rate lower than 2% at 50 ng l^{-1} and a false positive rate of less than 0.3%. Quantitative readings had a mean bias of +2 to 6 ng l^{-1} at 50 ng l^{-1} with a standard deviation of 5–8 ng l^{-1}. Based on the validation results, the test could be considered appropriate for milk screening prior to milk unload at dairies.

Keywords: aflatoxin M_1; raw milk; validation; European Union maximum limit; quantitative screening test

Introduction

Aflatoxins are natural toxins produced as a secondary product of *Aspergillus flavus* and/or *Aspergillus parasiticus* mould growth (Klich 2007). When mould growth occurs on feeds or grains, aflatoxin B_1 could be produced. Aflatoxin B_1 is the most toxic aflatoxin and a potent hepatocarcinogen (Wogan 1966; Carnaghan 1967; Wong & Hsieh 1976). Feeds and grains are generally screened in a range of 2 µg kg^{-1} aflatoxin B_1 for European Union (EU) Regulation No. 165/2010 and 20 µg kg^{-1} aflatoxin B_1 for the USFDA action level for human consumption and for dairy animals (USDA 2002).

When feed contaminated with aflatoxin B_1 is consumed by dairy cows, usually some 1–3% of the aflatoxin B_1 is excreted as aflatoxin M_1 into the milk (Veldman et al. 1992; Masoero et al. 2007). Aflatoxin M_1, which is the aflatoxin B_1 hydroxylated metabolite, is a less potent carcinogen but still classified as a group 1 carcinogen by the IARC (Wogan & Paglialunga 1974; Hsieh et al. 1986; Cullen et al. 1987; IARC 2012). There are no strategies for removal of aflatoxin M_1 from milk, hence attention has to be focused on methods to prevent or reduce mycotoxin formation at all stages in the milk production chain (van Egmond et al. 1997; Prandini et al. 2009). Pre-screening of feeds and grain prior to consumption could be a first control strategy to limit the amount of aflatoxin M_1 in milk. Because grain and feed screening is not always performed, screening of the milk supply on aflatoxin M_1 at the raw milk level prior to dairy intake is an important control check since the occurrence of mould growth and toxin production in grains and feeds is increasing due to the changing climatic conditions (Driehuis et al. 2010).

In some countries like Iran, Turkey, Pakistan and India, the levels of aflatoxin M_1 in milk often (30.8%, 47%, 81%, and 86%, respectively) exceeds the EU permissible level of 0.05 µg l^{-1} (Muhammad et al. 2010) due to the poor storage conditions along the animal feed chain which exacerbates the growth of moulds and consequently increases the concentration of mycotoxins in cow feed.

The established CODEX health level for aflatoxin M_1 in milk, and the US action level, is 0.5 µg l^{-1} or 500 ng l^{-1} (Codex 2001). In Europe, a maximum limit (ML) based on the ALARA (as low as reasonably achievable) principle is set for aflatoxin M_1 in raw milk at 0.05 µg l^{-1} or 50 ng l^{-1} (Commission Regulation (EU) No. 165/2010).

A variety of methods exist for evaluating aflatoxin M_1 in milk. One AOAC method is also the ISO method validated at the 80 ng l^{-1} level for reconstituted powdered milks (ISO/IDF 2007). This uses an immunoaffinity purification column followed by derivatisation and fluorescent HPLC detection. The repeatability at the lowest study concentration of 80 ng aflatoxin M_1 l^{-1} was 50 ng l^{-1} (CV = 62.5%). Due to the equipment costs and complexity of the method, the reference method has limited to no field

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applicability for the rapid detection of aflatoxin M_1. More rapid methods with high throughput using ELISA and lateral flow methods are employed for screening purposes. While many methods have been validated for the detection of aflatoxin B_1 in feeds and grain, only a few have been validated for aflatoxin M_1 in milk (Anfossi et al. 2010). Most of these methods are designed to screen aflatoxin M_1 in milk at USFDA-established action level and are hence not suitable for the European market. Other rapid tests are not available as a single test or the test protocol is too complex to be used for screening at the entry of the dairy. In other tests, e.g. Afla M1-V (Vicam), a filtration step is involved as sample pre-treatment. To our knowledge there are actually only two rapid tests on the market based on the lateral flow principle with no sample dilution or preparation for raw milk screening on aflatoxin M_1 at the 50 ng l^{-1} level, namely Aflasensor (Unisensor s.a.) and MRLAFMQ (Aflatoxin M1) Test (Charm Sciences Inc., Lawrence, MA, USA).

This study describes a dairy stakeholder requested independent laboratory evaluation of the MRLAFMQ (Aflatoxin M1) Test for detecting 50 ng l^{-1} of aflatoxin M_1 in milk and quantifying in a 15–75 ng l^{-1} range. The validation design performed at the Technology and Food Science Unit of the Institute for Agricultural and Fisheries Research (ILVO-T&V) is based on its applicability as a qualitative screening test according to Commission Decision 2002/657/EC and CRL Guidelines (CRL 2010) in order to check if the test is suitable as a raw milk screening test at 50 ng aflatoxin M_1 l^{-1}, the European ML. Additionally, because the method provides a semi-quantitative result, the data may be used to evaluate quantitative test parameters defined in Commission Decision 2002/657/EC to compare with other semi-quantitative methods. The test is typically used for testing farm tanks and truck loads of milk before the receipt of milk into dairies.

Materials and methods

Tests and equipment

MRLAFMQ tests (kit lot 009 (Exp. 09/2013) and lot 010 (Exp. 10/2013)) (Charm Sciences Inc., Lawrence, MA, USA), a 45°C ROSA incubator, and reader models ROSA Pearl and EZ were provided to ILVO-T&V. The MRLAFMQ Test for milk is a lateral flow method that works in 15 min with a single milk addition step. The test principle is similar to the optimised system described by Anfossi et al. (2010). However, it is different in the way it employs two competitive binding lines as well as a control line. This test is based on the competitive binding of aflatoxin M_1 in the milk with colloidal gold-antibody construct contained within a lateral flow device and solubilised by milk as it flows through the strip. When complete, two test lines and a control line are visible that are read by a reader using a line refractance algorithm to provide a quantitative test result in ng aflatoxin M_1 l^{-1}, also referred to as parts per trillion (ppt). The readers also employ a 40 ng l^{-1} qualitative negative/positive control limit to provide confidence that milk containing aflatoxin M_1 at 50 ng l^{-1} or greater is not accepted. Two reader models, the ROSA Pearl Reader and the EZ Reader, were evaluated in this study because both readers models are currently in use in dairies worldwide.

Reported values

Values reported in tables for spiked concentrations include mean, standard deviation (SD) and minimum and maximum values of the *n*-replicates.

Clean-up by immunoaffinity chromatography and determination by HPLC with fluorescence detection based on ISO 14501:2007 (ISO/IDF 2007). LOD = 1.5 ng aflatoxin M_1 l^{-1} and LOQ = 3 ng aflatoxin M_1 l^{-1}.

Reagents

Aflatoxin M_1 analytical standard of 10 µg ml^{-1} in acetronitrile (Supelco-46319-U Sigma-Aldrich, Bornem, Belgium) and further used for the preparation of an 1 µg aflatoxin M_1 ml^{-1} stock solution in phosphate buffer stored refrigerated for spiking raw and pasteurised milk.

Milk supply

The blank milk for spiking was raw commingled milk from local Belgian farms with a low aflatoxin M_1 background originating from at least four healthy cows in mid-lactation that were not treated with antibiotics or chemotherapeutics for at least 3 months and stored refrigerated for a maximum of 3 days. Pasteurised milk was whole milk from the Netherlands purchased at the supermarket. Farm and truck milk samples were from the Flemish milk control station Melkcontrolecentrum-Vlaanderen. Incurred individual farm silo milk samples were obtained from two Belgian farms with aflatoxin M_1 problems in the milk.

Results and discussion

Detection capability

Raw milk was spiked with aflatoxin M_1 at three levels: 25, 50 and 75 ng l^{-1} in seven different blank raw milks. Sixty replicates tested at each concentration were used based on the closeness of the predicted 95% sensitivity (concentration capabilities, CCβ) to the European maximum limit (ML) according to CRL Screening Test Guidelines (CRL 2010). The testing was performed over at least 7 days with the use of at least two different stock solutions for spiking. Two different lots of MRLAFMQ reagents were used for

Table 1. Spike milk sample results of MRLAFMQ by means of the ROSA Pearl Reader.

Concentration of aflatoxin M_1 (ng l^{-1})	Mean (ng l^{-1})[a]	SD (ng l^{-1})	HorRat (CV/PRSDR)[b]	Minimum reading	Maximum reading
Blank	2	4	–	0	26
25	25	6	0.80	13	36
50	52	6	0.38	38	68
75	76	8	0.35	54	91

Notes: [a]Number of replicates used to determine the mean for each concentration: $n = 59$ for blank, $n = 58$ for 25 ng aflatoxin M_1 l^{-1}, $n = 59$ for 50 ng aflatoxin M_1 l^{-1} and $n = 60$ for 75 aflatoxin M_1 ng l^{-1} because of some occasional invalid test developments in the $n = 60$ replicates.
[b]Ratio of the coefficient of variation (CV) (= SD/mean) and predicted relative standard deviation (PRSDR) calculated from the Horwitz equation PRSDR (%) = $2C(-0.15)$, where C is the spike concentration. CV is not calculated for the blank.

Table 2. Spike milk sample results of MRLAFMQ by means of the EZ Reader.

Concentration of aflatoxin M_1 (ng l^{-1})	Mean (ng l^{-1})[a]	SD (ng l^{-1})	HorRat (CV/PRSDR)[b]	Minimum reading	Maximum reading
blank	7	4	–	0	20
25	31	16	1.72	17	91
50	56	10	0.60	39	100
75	81	10	0.41	59	100

Notes: [a]Number of replicates used to determine the mean for each concentration: $n = 59$ for blank, $n = 58$ for 25 ng aflatoxin M_1 l^{-1}, $n = 60$ EZ for 50 ng aflatoxin M_1 l^{-1} and $n = 60$ for 75 aflatoxin M_1 ng l^{-1} because of some occasional invalid test developments in the $n = 60$ replicates.
[b]Ratio of the coefficient of variation (CV) (= SD/mean) and predicted relative standard deviation (PRSDR) calculated from the Horwitz equation PRSDR (%) = $2C(-0.15)$, where C is the spike concentration. CV is not calculated for the blank.

this validation and the results were measured using both the ROSA Pearl Reader and the EZ Reader. To verify the detection capability at 50 ng aflatoxin M_1 l^{-1}, the test and reader must provide a positive interpretation above the positive/negative cut-off, with a quantitative result of 40 ng aflatoxin M_1 l^{-1} or higher in at least 57 upon 60 tests. The performance of the test, for each reader, and at each concentration are given in Tables 1 and 2.

Using a value of 40 ng l^{-1} or greater as the cut-off for a qualitative positive result, 58 of the 59 fortified 50 ng aflatoxin M_1 l^{-1} samples for both the ROSA Pearl Reader and the EZ Reader were found as positive, which qualifies the MRLAFMQ for ML detection at 50 ng aflatoxin M_1 l^{-1}. There were 60 upon 60 positives at 75 ng l^{-1}. At 25 ng l^{-1} or 0.5 × ML, there were zero positives upon 58 replicates with the ROSA Pearl Reader and two positives upon 58 replicates with the EZ Reader, a false violative rate of 3.4%. These results indicate a high degree of discrimination between 0.5 × ML and ML. Blank milk tests demonstrated no positives of the 59 replicates with a maximum read of the ROSA Pearl Reader of 26 ng l^{-1} and of the EZ Reader of 20 ng l^{-1}.

Quantitative detection parameters

Quantitative aspects of the data support the qualitative test results. SDs of blank milk of 4 ng l^{-1} and 3× ±SD support an LOD between 14 and 19 ng l^{-1} for the ROSA Pearl Reader and the EZ Reader, respectively. LOQ and blank mean + 10× SD are between 42 and 47 ng l^{-1}, supporting a detection capability of 50 ng l^{-1}. The 50 ng l^{-1} mean values minus 2 × SD are 40 ng l^{-1} for the ROSA Pearl Reader and 36 ng l^{-1} for the EZ Reader and demonstrate that the 40 ng l^{-1} limit is providing about a 95% confidence in detecting 50 ng aflatoxin M_1 l^{-1} samples as positive.

The method LOD and LOQ as well as the SDs of quantification are consistent with semi-quantitative interpretation of test results. The estimated precision of a quantitative determination would be about 3 × SD or 15 ng l^{-1}, which is consistent with the 30% relative standard deviation (RSD %) of Anfossi et al. (2013) in the optimised lateral flow system. It is interesting to note that the SDs of lateral flow systems of about 5–8 ng kg^{-1} at 75 ng ml^{-1} are comparable with the ISO method repeatability, Sr = 0.005–0.008 µg kg^{-1}, at the lowest study concentration of 0.08 µg kg^{-1} (ISO/IDF 2007). Additionally HorRat values less than 1.0 at the 50 µg kg^{-1} level are an indicator of the acceptable repeatability of the method for raw milk analysis. These results indicate that the lateral flow method might be useful as a dairy screening method for quantitative determination consistent with subsequent official methods for legal action and milk rejection. Further collaborative study according to international protocol to establish inter-

laboratory repeatability and intra-laboratory reproducibility at 50 ng aflatoxin M_1 l^{-1} and lower are needed to compare different semi-quantitative methods.

Selectivity

The selectivity of the method is its ability to distinguish the target analyte, aflatoxin M_1, from other unrelated compounds, such as antibiotics; other unrelated mycotoxins, such as ochratoxin A, zearalenone, deoxynivalenol and fumonisin B1; and analogous compounds such as aflatoxin M_2. Different compounds were evaluated at 10 × MRL to determine if there was any interference. If interference did occur, the levels were adjusted to determine the percentage of cross reactivity.

The results in Table 3 indicate no interference in interpretation or reading with any unrelated antibiotic or mycotoxin. Aflatoxin M_2 did show about 20% cross-reactivity in quantification and began to produce positive interpretation at about 500–600 ng l^{-1} in both readers. The MRLAFMQ is highly selective for aflatoxin M-related compounds.

Repeatability

The repeatability of the reader was evaluated by measuring in duplicate (removing and replacing into the reader) 20 different strips obtained after the testing of blank, low positive 25 ng aflatoxin M_1 l^{-1}, and high positive 75 ng aflatoxin M_1 l^{-1}. The test repeatability was checked by randomly analysing 15 samples in duplicate for blank, low positive 25 ng aflatoxin M_1 l^{-1}, and high positive 75 ng aflatoxin M_1 l^{-1} milk. Differences between duplicate reader and test results were squared and SD are presented in Tables 4 and 5 for the reader and test, respectively. Results indicate that reader variation is less than the test or assay variation. Reader variation of the ROSA Pearl Reader has SD of differences less than 1 at all concentrations, while the EZ Reader had a slightly higher variation with SD less than 2 at all concentrations. The test or assay

Table 4. Reader repeatability of MRLAFMQ.

Concentration of aflatoxin M_1 (ng l^{-1})	ROSA Pearl Reader, SD of the square of pair differences	EZ Reader, SD of the square of pair differences
Blank	0.50	0.71
25	0.88	1.72
75	0.77	1.77

Table 5. Test repeatability of MRLAFMQ.

Concentration of aflatoxin M_1 (ng l^{-1})	ROSA Pearl Reader, SD of the square of pair differences	EZ Reader, SD of the square of pair differences
blank	1.72	2.83
25	4.53	4.06
75	6.11	7.00

Table 3. Test selectivity of MRLAFMQ test for aflatoxin M_1.

Chemical compound (concentration in ng l^{-1})	EZ Reader		ROSA Pearl Reader	
	Mean (ng l^{-1})	Result	Mean (ng l^{-1})	Result
Benzylpenicillin (40)	2	Negative	0	Negative
Cefalonium (200)	5	Negative	5	Negative
Oxytetracycline (1,000)	0	Negative	0	Negative
Erythromycin (400)	0	Negative	2	Negative
Neomycin (15,000)	2	Negative	5	Negative
Enrofloxacin (1,000)	0	Negative	0	Negative
Sulfadiazine (1,000)	4	Negative	4	Negative
Trimethoprim (500)	0	Negative	1	Negative
Dapson (50)	5	Negative	0	Negative
Ochratoxin A (500)	4	Negative	1	Negative
Zearalenone (250)	0	Negative	5	Negative
Deoxynivalenol (500)	3	Negative	5	Negative
Fumonisin B_1 (500)	4	Negative	1	Negative
Aflatoxin M_2 (100)	22	Negative	17	Negative
Aflatoxin M_2 (200)	19	Negative	22	Negative
Aflatoxin M_2 (300)	27	Negative	27	Negative
Aflatoxin M_2 (400)	24	Negative	26	Negative
Aflatoxin M_2 (500)	37	Negative	39	Negative/pos
Aflatoxin M_2 (600)	43	Negative/positive	41	Negative/positive

Note: Data are the mean (ng l^{-1}) of $n = 2$ tests and the interpretation is based on a 40 ng l^{-1} cut-off for each reader type.

variation is higher than the reader variation and the SD are similar to the SD of fortified sample experiments, suggesting that the test-to-test variation is the major contributing factor affecting result variance. Both the ROSA Pearl Reader and the EZ Reader had similar test variance SD at all test concentrations. The two readers can be considered to give equivalent test variation. This result supports the data of the fortified experiment that the EZ Reader is calibrated with a 5 ng l^{-1} higher positive bias and that this bias is consistently reflected at all four study concentrations.

Milk sample screening and false positive results

The incidence of aflatoxin M_1 contamination of milk farm tanks and milk bulk tanks is of interest based on the discovery of contamination of feed originating from Eastern Europe with traces found in the milk supply (Epi South 2013). This contaminated feed was used in some Western European countries. This study found that the baseline levels of aflatoxin M_1 in milk from Belgium and the Netherlands were below the LOD, 14–19 ng l^{-1}. This evaluation tested the MRLAFMQ-positive rate with 123 frozen and thawed farm blank milk samples and 131 fresh blank truck bulk tank milk samples. The evaluation also evaluated 20 powder milk samples rehydrated to 10% solids, pH balanced and centrifuged prior to testing. Since these milk samples have an unknown history, if positive they were also tested with HPLC methods to determine method agreement and if the MRLAFMQ results were correct. All samples evaluated tested negative on both reader types; these results are summarised in Table 6. There was one truck sample that tested positive with the ROSA Pearl Reader, but this result was not confirmed on a duplicate retest. There were five farm milk samples that tested positive by the EZ Reader due to positioning errors corrected on reinsertion and excluded from Table 6.

These results indicate that the false positive incidence of the MRLAFMQ is about 1 upon 274 tests, or about 0.3%. Care should be taken to insert strips properly into the EZ Reader when used in the read-only mode. These results are consistent with antibiotic screening test validations and appropriate for farm and tanker/truck screening (Reybroeck & Ooghe 2012). The results also indicated that the majority of farm and tanker milk tested from Belgium and the Netherlands region is below the LOD by the MRLAFMQ Test.

Some incurred individual farm silo milk samples were tested by both the MRLAFMQ and reference HPLC-affinity fluorescence detection method (ISO/IDF 2007). In general there was a good agreement (compliant/non-compliant) between the results obtained with both test methods, indicating that the MRLAFMQ method is also working comparatively with the reference method with incurred samples.

Table 6. Summary of negative and positive results with different farm milk, tanker/truck milk and reconstituted milk powder samples.

	Aflatoxin M_1 (ng l^{-1})	
	ROSA Pearl Reader	EZ Reader
Number of farm milk samples	131	126[a]
Number of initial positive	0	0[a]
Number confirmed positive	0	0
Number of tanker/truck milk samples	123	123
Number of initial positive	1	0
Number confirmed positive	0	0
Number powder milk samples	20	20
Number of initial positive	0	0
Number confirmed positive	0	0
Total initial positives	1	0[a]
Total confirmed positives	0	0
Total initial negative results	273	269[a]
Total confirmed negatives	274	269[a]
Mean ± SD of farm tanks	9 ± 7	16 ± 12
Minimum reading	0	0
Maximum reading	34	34[a]
Mean ± SD of truck tanks	10 ± 7	12 ± 5
Minimum reading	0	1
Maximum reading	41	31

Note: [a]Five farmer samples are not reported because they tested positive due to mis-insertion into the EZ Reader. They immediately tested negative on strip reinsertion into the reader.

Ruggedness

The test performance under assay variance conditions was evaluated using pipette variances of 330 µl (high) and 270 µl (low) and with different milk temperatures, 3, 10, 15 and 20°C. Three replicates were evaluated using negative and positive milk doped with aflatoxin M_1 at 25 and 50 ng l^{-1}. The minimum and maximum results and the mean reading are presented in Tables 7 and 8.

Milk volume did not significantly affect negative or positive results. One false negative result occurred with the ROSA Pearl Reader at low volume dispense, and one false violative result occurred with the high volume dispensed on the EZ Reader. Similarly, milk temperature did not have an effect on MRLAFMQ results. There was one false negative result with the ROSA Pearl Reader at 15°C. None of the results is outside the normal spiking population of 60 data points and therefore the results are not considered significant indicators of perturbation. Similar readings and positive biases were produced with both readers in comparison with the spike data.

Interferences

The influence of milk compositional components or milk quality, $>10^6$ somatic cells ml^{-1}, $>5 \times 10^5$ bacteria ml^{-1}, low and high fat, low and high protein, and low and high

Table 7. Effect of change in milk volume on MRLAFMQ readings.

	ROSA Pearl Reader			EZ Reader		
	Milk volume			Milk volume		
Sample	300 µl	270 µl	330 µl	300 µl	270 µl	330 µl
Blank milk						
Mean	8	5	4	12	9	10
Minimum	3	0	2	8	6	9
Maximum	15	8	8	14	16	10
Aflatoxin M_1 25 ng l^{-1}						
Mean	24	27	20	29	29	30
Minimum	22	19	15	26	24	26
Maximum	26	34	23	32	31	82
Aflatoxin M_1 50 ng l^{-1}						
Mean	52	48	53	55	51	61
Minimum	47	39	51	53	49	54
Maximum	59	52	56	58	54	67

Table 8. Effect of the milk temperature on MRLAFMQ readings.

	ROSA Pearl Reader				EZ Reader			
	Milk temperature				Milk temperature			
	3°C	10°C	15°C	20°C	3°C	10°C	15°C	20°C
Blank milk								
Mean	1	0	0	2	6	4	6	6
Minimum	0	0	0	0	4	1	3	5
Maximum	3	1	0	5	9	9	9	7
Aflatoxin M_1 25 ng l^{-1}								
Mean	27	23	25	24	29	30	31	34
Minimum	24	21	24	19	26	27	30	31
Maximum	31	24	26	27	31	32	34	38
Aflatoxin M_1 50 ng l^{-1}								
Mean	66	55	46	51	57	64	60	61
Minimum	62	53	37	46	51	62	55	54
Maximum	72	57	54	61	62	66	63	68

pH were compared with blank milk of normal quality/composition, and spiked near CCβ with aflatoxin M_1 at 50 ng l^{-1}. Ten replicates of each milk type were performed. Mean, minimum and maximum values are shown graphically for the EZ Reader in Figures 1 and 2 for blank milk and milk spiked with aflatoxin M_1, respectively. The results for blank and spike milk for the ROSA Pearl reader are not shown. The results from the compositional/quality analysis show that compositional milk quality aspects did not influence the blank results but did have the effect of lowering the mean positive result in the cases of high pH, low and high fat, and low and high protein. These effects are likely due to slow flow of the milk through the test. The figures show that high somatic cell and bacteria have little effect on the results. The likely causes of false negative results with the abnormal milk compositional variants are a slight sensitivity shift due to flow differences.

The detection capability of the MRLAFMQ method was also evaluated in four different doped homogenised pasteurised whole-milk samples over 4 days. The performances of the test, for each reader, at each concentration are given in Tables 9 and 10. The results of pasteurised milk experiments are similar to the raw milk spike results, with a slightly higher positive bias on all the tested concentrations. There were no false negative results at 50 ng aflatoxin M_1 l^{-1} and 75 ng aflatoxin M_1 l^{-1}. There were no false positive results with negative milk. At 25 ng aflatoxin M_1 l^{-1} there were two false violative results using the ROSA Pearl Reader and five false violative results using the EZ

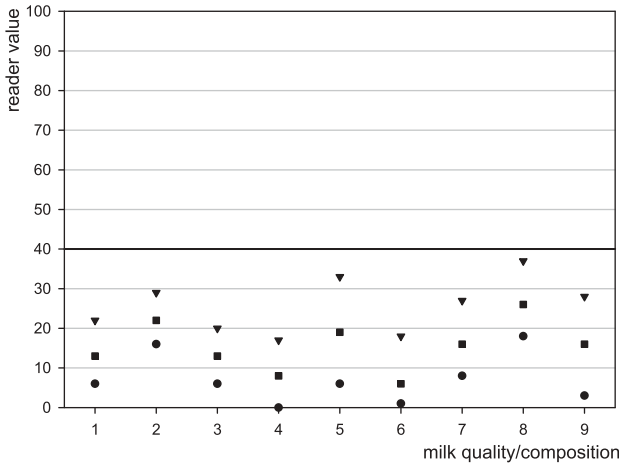

Figure 1. Effect of milk composition or quality effects on the screening of blank milk using MRLAFMQ and EZ Reader. Maximum reading (▼), average reading (■), minimum reading (●), control point (40 ng l^{-1}) dividing positive from negative (——); 1 = reference: normal raw cows' milk, 2 = somatic cell count > 10^6 ml^{-1}, 3 = high bacterial count (>5 × 10^5 ml^{-1}), 4 = low fat content (<2 g 100 ml^{-1}), 5 = high fat content (>6 g 100 ml^{-1}), 6 = low protein (<3 g 100 ml^{-1}), 7 = high protein (>4 g 100 ml^{-1}), 8 = low pH (6.0), 9 = high pH (7.5).

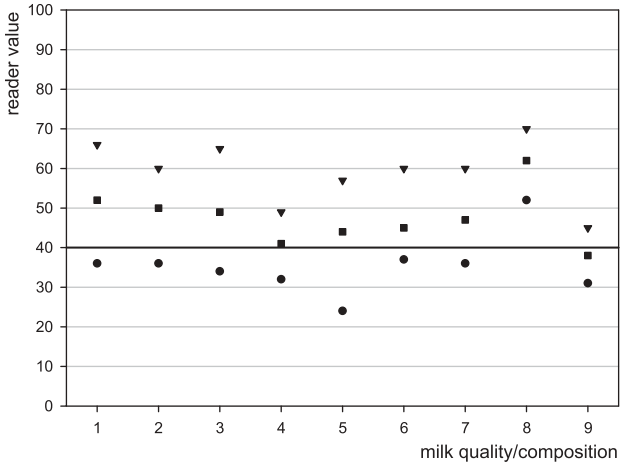

Figure 2. Effect of milk composition or quality on the detection of 50 ng l^{-1} aflatoxin M_1 in milk using MRLAFMQ and EZ Reader. Maximum reading (▼), average reading (■), minimum reading (●), control point (40 ng l^{-1}) dividing positive from negative (——); 1 = reference: normal raw cows' milk, 2 = somatic cell count > 10^6 ml^{-1}, 3 = high bacterial count (>5 × 10^5 ml^{-1}), 4 = low fat content (<2 g 100 ml^{-1}), 5 = high fat content (>6 g 100 ml^{-1}), 6 = low protein (<3 g 100 ml^{-1}), 7 = high protein (>4 g 100 ml^{-1}), 8 = low pH (6.0), 9 = high pH (7.5).

Reader. It is important that non-raw matrices tested with the method are internally validated by the laboratory performing the method to assure the performance and reliability of the test results since these other dairy matrices can have a different flow rate as compared with raw milk.

Table 9. Pasteurised whole-milk spike sample results of MRLAFMQ by means of the ROSA Pearl Reader.

Concentration of aflatoxin M_1 (ng l^{-1})	Mean (ng l^{-1})	SD (ng l^{-1})	Minimum reading	Maximum reading
Blank	11	6	0	21
25	31	5	15	42
50	56	6	45	66
75	77	8	59	95

Table 10. Pasteurised whole-milk spike sample results of MRLAFMQ by means of the EZ Reader.

Concentration of aflatoxin M_1 (ng l^{-1})	Mean $N = 60$ (ng l^{-1})	SD (ng l^{-1})	Minimum reading	Maximum reading
Blank	11	6	0	24
25	33	6	21	45
50	58	8	42	79
75	78	7	63	96

The MRLAFMQ Test is a test claimed for raw cows' milk. It is not claimed and has not been validated with goats' and ewes' milk nor powdered, UHT or heat-treated milk. Additional testing of the effect of composition looked at the influence of these different types of milks: UHT, sterilised, reconstituted powder, frozen–thawed, goats', ewes' and mares' milk. In this evaluation 10 negative raw milk and 10 negative heat-treated milk samples, and each sample spiked with aflatoxin M_1 at 50 ng l^{-1} were tested and evaluated in each reader. Minimum, maximum and mean obtained in the ROSA Pearl Reader are plotted in Figures 3 and 4 for blank milk and for milk spiked with aflatoxin M_1 at 50 ng l^{-1}, respectively. Comparable results were obtained in an EZ Reader (figures not shown).

Results are generally biased low, or high, depending on milk type. UHT milk displayed a higher average result for both blank and spiked milk. Sterilised and powdered milk had lower spiked positive averages. Thawed milk had more erratic minimum and maximum compared with normal milk. Goats' milk was biased positive, while mares' milk was biased negative. Ewes' milk is not reported as it did not produce valid results due to flow issues.

The MRLAFMQ Test is influenced by milk composition, and care should be taken to validate and calibrate instrumentation if other matrices than raw cows' milk are tested.

Lot differences

The following samples were analysed at the same time with two different batches of MRLAFMQ reagents (Lot 010 (Exp. Sep. 2013) and Lot 011-EZ (Exp. Oct. 2013)):

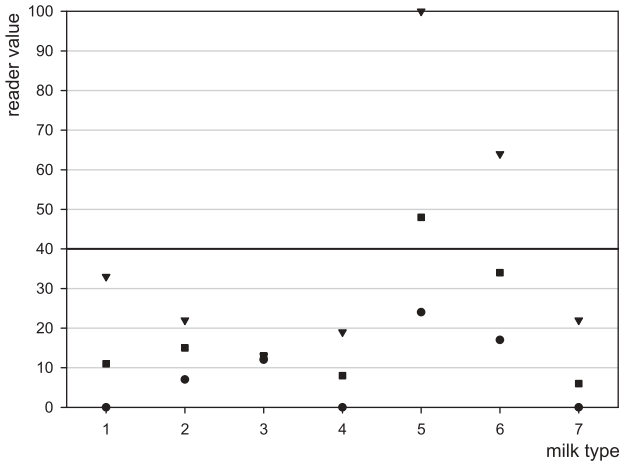

Figure 3. Screening of different blank milk types using MRLAFMQ and ROSA Pearl Reader. Maximum reading (▼), average reading (■), minimum reading (●), control point dividing positive from negative (——) ; 1 = reference: normal raw cows' milk, 2 = UHT milk, 3 = sterilised milk, 4 = reconstituted powder, 5 = frozen–thawed, 6 = goats' milk, 7 = mares' milk.

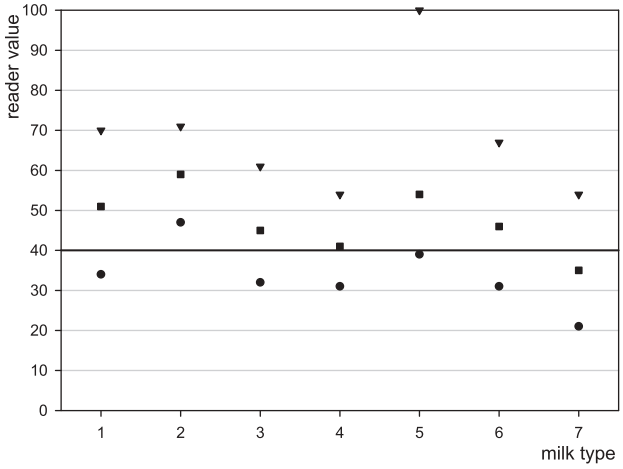

Figure 4. Detection of 50 ng l^{-1} aflatoxin M_1 in different milk types using MRLAFMQ and ROSA Pearl Reader. Maximum reading (▼), average reading (■), minimum reading (●), control point dividing positive from negative (——) ; 1 = reference: normal raw cows' milk, 2 = UHT milk, 3 = sterilised milk, 4 = reconstituted powder, 5 = frozen–thawed, 6 = goats' milk, 7 = mares' milk.

- Blank milk (antibiotic-free raw milk) (20 samples).
- Raw milk spiked with 25 ng l^{-1} aflatoxin M_1 (20 samples).
- Raw milk spiked with 50 ng l^{-1} aflatoxin M_1 (20 samples).
- Raw milk doped with 75 ng l^{-1} aflatoxin M_1 (20 samples).

The results are shown in Table 11. Both readers show similar performance for the two lots. In general comparable results were obtained for the two lots in that the differences between mean values were within 2–6 and 1–6 ng aflatoxin M_1 l^{-1} for EZ and ROSA Pearl Reader, respectively. Likewise, the maximum and minimum extremes were within a few ng aflatoxin M_1 l^{-1} of each other. The exception of this statement is with MRLAFMQ Lot 011 at 75 ng aflatoxin M_1 l^{-1}, which displayed a more negative minimum value, by about 10 ng aflatoxin M_1 l^{-1}, and in one case gave a false negative result with the ROSA Pearl Reader. There were no false negative results from any other of the positive spiked (50 and 75 ng aflatoxin M_1 l^{-1}) samples. There was one false positive result with blank milk analysed with the EZ Reader for both MRLAFMQ lots 010 and 011 and on reinsertion it was analysed as negative. This is likely a positional error discussed earlier in the false positive-selectivity paragraph.

Stability of result

The stability in readings was also evaluated by control chart plotting daily control performance and calibration data over the 2-month evaluation period. Daily negative controls and 50 ng aflatoxin M_1 l^{-1} Charm-positive controls for EZ Reader are depicted in Figure 5. There were no false positive results and one false negative Charm-positive control with the EZ reader and three false negative Charm-positive controls with the ROSA Pearl Reader (figures not shown). In addition, milk spiked with aflatoxin M_1 standard (diluted from Supelco 10 µg aflatoxin M_1 ml^{-1}) was monitored and gave results similar to the positive control standard, which are depicted for the EZ Reader in Figure 6. There was one false negative with the 50 ng aflatoxin M_1 l^{-1} spiked raw milk in each reader (the figure for the ROSA Pearl Reader is not shown). False-negative positive controls were followed up with true positive results to verify equipment operation before continuing evaluation.

In summary, the MRLAFMQ is a very selective lateral flow test for commingled raw milk that detects aflatoxin M_1 at 50 ng l^{-1} in 15 min. The method is qualitative using a 40 ng l^{-1} limit and demonstrated detection capability at 50 ng l^{-1}, a low <3.4% false violative rate at 25 ng l^{-1}, and a false positive rate of 0.3% While the method did demonstrate detection capability at EU ML with <2% false negative results, by lowering the cut-off to 38 ng l^{-1} no false-negatives could be obtained providing additional confidence and robustness at detecting 50 ng l^{-1} while not increasing false positive incidence. The method was also tested and is applicable to pasteurised milk, but based on a testing anomaly with a particular type of pasteurised milk it is recommended that laboratories test and specifically qualify non-raw milk type matrices before routine use of the assay.

The MRLAFMQ method provides a quantitative value with an LOD of 14–19 ng aflatoxin M_1 l^{-1}, an LOQ of 45–50 ng aflatoxin M_1 l^{-1}, and SDs of readings of about

Table 11. Testing of blank milk, milk spiked with 25 ng l^{-1} aflatoxin M_1, 50 ng l^{-1} aflatoxin M_1 and 75 ng l^{-1} aflatoxin M_1 using two different lots of MRLAFMQ.

	ROSA Pearl Reader						EZ Reader					
	Lot 010			Lot 011			Lot 010			Lot 011		
Sample	Mean	Minimum	Maximum	Mean	Minimum	Maximum	Mean	Minimum	Maximum	Mean	Minimum	Maximum
Blank milk	2	0	9	1	0	7	7	0	43	3	0	40
25 ng l^{-1} Aflatoxin M_1	30	18	40	24	16	34	32	23	43	26	17	35
50 ng l^{-1} Aflatoxin M_1	56	40	89	55	41	81	60	40	77	58	41	74
75 ng l^{-1} Aflatoxin M_1	77	49	95	74	38	100	82	59	100	77	44	98

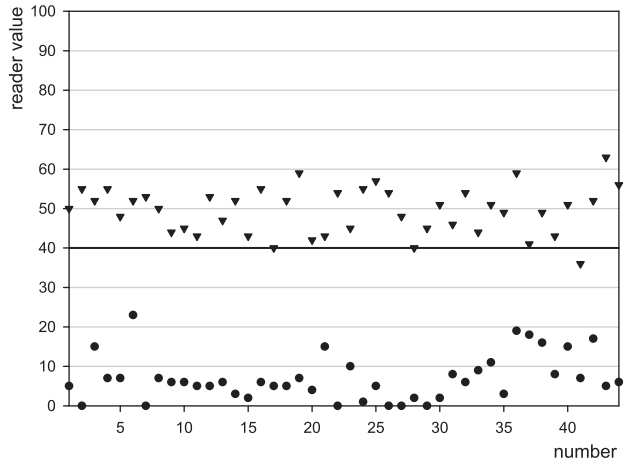

Figure 5. Results for daily blank samples (●) and Charm-positive control (▼) by means of the EZ Reader.

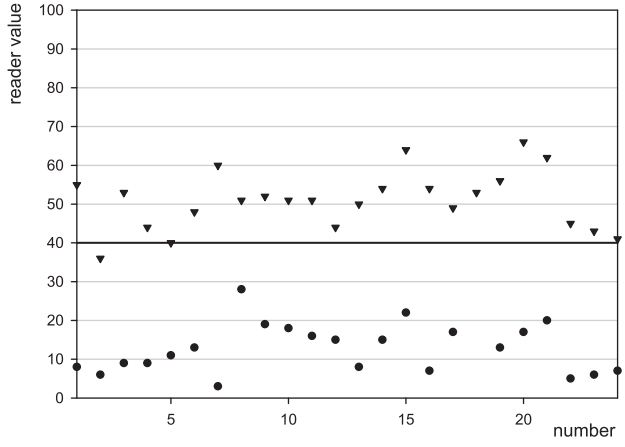

Figure 6. Results for daily blank samples (●) and 50 ng l^{-1} aflatoxin M_1 spiked standard (▼) by means of the EZ Reader.

5–10 ng l^{-1}. The method was evaluated for influences from compositional components or milk quality and could result in false positive results when testing frozen–thawed samples and goats' milk. The method is applicable to normal raw cows' milk as abnormal fat, protein and pH levels caused loss of detection capability.

The method was easily performed using the existing ROSA Pearl Reader and the new EZ Reader equipment in use at dairy laboratories. The method was robust to milk temperature variations and to the correct amount of milk to within ±10% of the target level.

The MRLAFMQ method meets screening test specifications of low false negatives and positives, low false violatives, and detection capability of aflatoxin M_1 at 50 ng ml^{-1} which is the EU ML. This indicates the method can reliably be used at dairy milk receipt and in trade to verify milk is free of aflatoxin M_1 below levels of concern. Based on the validation data and the results for the incurred samples, the

MRLAFMQ was accepted by the Belgian Federal Agency for the Safety of the Food Chain as a method that could be used for testing farm silo milk to re-allow milk collection after the farm was put in 'quarantine' due to the delivery of milk containing aflatoxin M_1 above the ML. Routine use of the method can allow for quantification above the LOD and below the LOQ; this is useful for farm feed remedial action before milk becomes actionable and proactively maintain ALARA levels. An additional international collaborative study to determine method precision parameters is warranted based on these study results.

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