FOOD CHEMICAL CONTAMINANTS

# Interlaboratory Study of the Charm ROSA Safe Level Aflatoxin M1 Quantitative Lateral Flow Test for Raw Bovine Milk

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**An interlaboratory study of 21 public health, state agriculture, and industry laboratories in the United States tested raw commingled bovine milk containing aflatoxin M1 using the Charm Rapid One Step Assay (ROSA) Safe Level Aflatoxin M1 Quantitative lateral flow method. Blind coded sample pairs were fortified with 0, 300, 350, 400, 450, 500, and 550 parts per trillion (ppt) aflatoxin M1. A ROSA reader quantitatively interpreted test strips with ppt readings. Readings 400 ppt were interpreted as negative, and readings >400 ppt were interpreted as positive. Initial positive samples were subsequently assayed 2 additional times. If both retest results were >400 ppt, the sample was called positive/ actionable relative to U.S. and Codex levels, 500 ppt. The concentration of 400 ppt was chosen for the positive/negative interpretation to provide 90% sensitivity with 95% confidence at the 500 ppt legislative level. The combined false negative rate was <5% (4 of 83) for samples at 500 and 550 ppt. The false violatives at 0, 300, 350, 400, and 450 ppt (*n* = 42 at each level) were 0, 0, 21, 14, and 93%, respectively. The 90% positive concentration with 95% confidence was 503 ppt by probit analysis. The average intralaboratory repeatability was 11% and average interlaboratory reproducibility was 13% for the fortified sample pairs. High-performance liquid chromatography analysis of the study samples by 5 laboratories showed 38% false negatives with the 500 and 550 ppt samples, and a 0% false-violative rate with samples less than the 500 ppt action level.**

The U.S. and Codex established action level for aflatoxin M1 in milk is 500 parts per trillion (ppt; 1, 2). Rapid screening methods such as enzyme-linked immunosorbent assay, immunoaffinity, and lateral flow tests are used by industry and state laboratories for screening milk samples (3, 4). Positive samples may require further analysis by validated methods such as the officially approved high-performance liquid chromatography (HPLC) methods for aflatoxin M1 in milk (5–7). With any methodology, there are concerns about the sensitivity, precision, and reproducibility of the method and the subsequent rate of false-positive, false-violative (positive test result with non-actionable levels in the sample), and false-negative results (8). Rapid screening methods need to provide detection at the action level but not be overly sensitive as to cause the loss of milk due to false violatives (9).

The Charm Safe Level Aflatoxin M1 Quantitative (SLAFMQ) test is a colloidal gold lateral flow immunoassay. Aflatoxin M1 in a milk sample competes with the antibody gold beads for binding to 2 test lines. Remaining unbound binder forms on the control line. The test and control lines are compared with a reflectance reader, and a ppt concentration is determined with an algorithm. A negative interpretation with a reading of 400 ppt and a positive interpretation with a reading >400 ppt was designed to detect 500 ppt, the U.S. and Codex violative level at 90% positive with 95% confidence. Retesting an initial positive sample 2 additional times was a confirmation step to reduce false-violative samples.

The purpose of this interlaboratory study was to determine the multilaboratory performance characteristics of the SLAFMQ test in raw milk and to compare these results with validated HPLC methods. This study was organized by regional state health laboratories and Charm Sciences. Data were sent to an independent third party who sent results to Charm Sciences for computation.

## Methods

### *SLAFMQ Procedure*

Equipment and reagents were supplied to volunteer laboratories along with 4 prestudy samples at 0, 300, 400, and 500 ppt to familiarize the laboratory analysts with the SLAFMQ lateral flow method. Rapid One Step Assay (ROSA) Reader v.1.08.54 was used for analysis. The SLAFMQ method is as follows: (*1*) Pipet 300 L cold (0–7 C) dilution buffer into microcentrifuge tube. (*2*) Mix

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**Table 1. Testing scenarios and interpretation of test results**

| Initial test result | Retest No. 1 result | Retest No. 2 result | Interpretation |
|---|---|---|---|
| 400 ppt or less–negative | NA[a] | NA | Negative/non-actionable |
| 401 ppt or greater–positive[b] | 400 ppt or less–negative | 400 ppt or less–negative | Negative/non-actionable |
| 401 ppt or greater–positive[b] | 400 ppt or less–negative | 401 ppt or greater–positive[b] | Negative/non-actionable |
| 401 ppt or greater–positive[b] | 401 ppt or greater–positive[b] | 400 ppt or less–negative | Negative/non-actionable |
| 401 ppt or greater–positive[b] | 401 ppt or greater–positive[b] | 401 ppt or greater–positive[b] | Positive/actionable, 500 ppt or greater |

[a] NA = Not applicable to retest an initial test result 400 ppt or less.
[b] Positive interpretation by ROSA reader.

raw milk, and pipet 300 L into the microcentrifuge tube. Cap and shake mixture vigorously for 5 s. Keep cold at 0–7 C. (*3*) Place SLAFMQ strip in 45 2 C incubator, peel open sample compartment, pipet 300 L of the above mixture into compartment, reseal strip, and close incubator lid, which starts an 8 min timer. (*4*) After 8 min, remove SLAFMQ strip and visually inspect control line for even development to determine that the test result is valid. (*5*) Insert valid strip into ROSA reader for 5 s analysis. The reader displays the determined ppt concentration and interprets a value >400 ppt as positive and a value 400 ppt as negative.

For the study, testers retested any positive sample 2 more times. If both the additional tests were positive, the sample was positive/actionable. If either retest was negative, the sample was negative/non-actionable.

### *Blind Study Sample Preparation*

Two raw milk samples from farm bulk tanks were analyzed to contain <50 ppt aflatoxin M1 using a lateral flow test, Maximum Residue Level for Aflatoxin M1 method (LF-MRLAFM, Charm Sciences), to screen European Union maximum residue levels of aflatoxin M1, 50 ppt. Sigma aflatoxin M1 standard (Cat. No. 49319-U) was made to a stock concentration of 5 g aflatoxin M1/mL (in acetonitrile) and verified by a Varian spectrophotometer at $A_{350nm}$ = 0.3033. Qualified raw milk was prepared to contain 300, 400, and 500 ( 3% based on volumetric and pipet error) ppt from the aflatoxin M1 standard. Another qualified raw milk sample was prepared to contain 0, 350, 450, and 550 ( 3%) ppt. Milk samples in 5 mL portions were sealed under nitrogen in glass vials. Duplicate samples were blind coded, shipped on ice, and tested by participants within 1 week. Four laboratories with American Oil Chemist proficiency certification or FDA-CFSAN certification and one noncertified laboratory analyzed samples by AOAC HPLC methods.

### *Statistical Design*

The design of the study was based on international interlaboratory study protocol ISO-5525 (10). All laboratories tested each sample once and reported these initial results. Data analysis for outliers and statistical parameters for repeatability (r) and reproducibility (R) were calculated with initial results of the blind coded sample pairs according to ISO-5525-2. Samples with an initial positive result were assayed 2 more times, and samples were interpreted positive/actionable or negative/non-actionable as described in SLAFMQ procedure. The 90% positive concentrations with 95% confidence level were determined from dose-response versus concentration curves. The SLAFMQ method positives and initial test positives, per total number of samples tested at each concentration, were analyzed by XL-Stat™ probit analysis with the 95% confidence value converted to one-tail (11). The National Conference of Interstate Milk Shipments (NCIMS) has used similar statistics to validate that antibiotic screening tests have 90% positive concentrations with 95% confidence at or below U.S. safe levels/tolerances (12). To maintain proper blind study protocol, the data from each participant were forwarded to the Texas Department of State Health Services, an independent third party that was not involved in sample preparation, for collection, scoring, and decoding.

## Results and Discussion

The SLAFMQ method testing scenarios for the initial test result and subsequent retests, and the interpretation of these results in terms of negative/non-actionable and positive/actionable, are shown in Table 1. Only a sample that tested positive on the initial test and positive on the 2 subsequent retests was considered to be at 500 ppt or greater and interpreted to be a positive/actionable sample. The initial test results in ppt for all 294 samples analyzed are presented in Table 2 and in Figure 1. The samples that tested >400 ppt on the initial test were retested 2 additional times, and these results are presented in Table 3. The interpretation of all samples from data from Tables 2 and 3 in terms of negative/non-actionable and positive/actionable is presented in Table 4. Samples that were positive at 350, 400, and 450 ppt can be considered false-violative samples since they were found positive/actionable by the method but contained aflatoxin M1 less than the 500 ppt violation/action level (9). Outliers were determined by Cochran and Grubbs analysis with 1 Cochran outlier found in each of the 0 ppt samples and

**Table 2. Initial reading of blind coded samples using charm SLAFMQ assay[a]**

| ID | Added concn, ppt | Laboratory | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| 1 | 450 | 503[b] | 523[b] | 489[b] | 420[b] | 499[b] | 515[b] | 691[b] | 493[b] | 550[b] | 478[b] | 558[b] | 574[b] | 536[b] | 467[b] | 468[b] | 527[b] | 464[b] | 495[b] | 447[b] | 422[b] | 711[b] |
| 2 | 300 | 238 | 250 | 326 | 263 | 303 | 282 | 317 | 278 | 299 | 309 | 251 | 304 | 246 | 290 | 307 | 248 | 290 | 329 | 312 | 221 | 413[b] |
| 3 | 350 | 352 | 457[b] | 387 | 304 | 400 | 410[b] | 457[b] | 434[b] | 386 | 422[b] | 471[b] | 356 | 408[b] | 413[b] | 357 | 260 | 324 | 401[b] | 414[b] | 377 | 580[b] |
| 4 | 500 | 629[b] | 515[b] | 443[b] | 567[b] | 527[b] | 453[b] | 533[b] | 460[b] | 522[b] | 512[b] | 524[b] | 502[b] | 452[b] | 565[b] | 399 | 479[b] | 477[b] | 437[b] | 431[b] | 451[b] | 588[b] |
| 5 | 300 | 284 | 310 | 251 | 316 | 292 | 289 | 285 | 297 | 350 | 264 | 316 | 234 | 320 | 307 | 269 | 235 | 264 | 297 | 298 | 253 | 412[b] |
| 6 | 450 | 436[b] | 522[b] | 425[b] | 526[b] | 489[b] | 411[b] | 522[b] | 475[b] | 515[b] | 551[b] | 512[b] | 488[b] | 553[b] | 453[b] | 504[b] | 478[b] | 440[b] | 511[b] | 472[b] | 489[b] | 614[b] |
| 7 | 550 | 612[b] | 589[b] | 573[b] | 662[b] | 642[b] | 531[b] | 618[b] | 619[b] | 591[b] | 653[b] | 677[b] | 632[b] | 658[b] | 605[b] | 501[b] | 668[b] | 549[b] | 577[b] | 349[c] | 574[b] | 638[b] |
| 8 | 400 | 340 | 405[b] | 436[b] | 419[b] | 341 | 344 | 348 | 365 | 410[b] | 382 | 464[b] | 375 | 487[b] | 445[b] | 350 | 442[b] | 376 | 381 | 275 | 330 | 503[b] |
| 9 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 106[c] | 14 | 0 | 0 | 0 | 3 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 9 | 0 | 14 | 11 | 0 | 0 | 0 | 0 | 0 | 0 | 55[d] | 1 | 0 | 0 | 0 | 30 | 0 | 4 |
| 11 | 350 | 412[b] | 355 | 400 | 98 | 378 | 291 | 443[b] | 421[b] | 408[b] | 361 | 470[b] | 391 | 410[b] | 435[b] | 225 | 427[b] | 412[b] | 464[b] | 360 | 320 | 461[b] |
| 12 | 500 | 474[b] | 602[b] | 507[b] | 309[d] | 475[b] | 498[b] | 511[b] | 553[b] | 506[b] | 496[b] | 483[b] | 525[b] | 533[b] | 450[b] | 493[b] | 447[b] | 436[b] | 517[b] | 491[b] | 437[b] | 561[b] |
| 13 | 400 | 411[b] | 442[b] | 353 | 375 | 326 | 363 | 408[b] | 402[b] | 401[b] | 333 | 427[b] | 450[b] | 440[b] | 478[b] | 336 | 429[b] | 406[b] | 434[b] | 367 | 390 | 370 |
| 14 | 550 | 550[b] | 576[b] | 606[b] | 639[b] | 604[b] | 582[b] | 591[b] | 634[b] | 557[b] | 568[b] | 637[b] | 569[b] | 575[b] | 623[b] | 523[b] | 640[b] | 518[b] | 697[b] | 603[b] | 614[b] | 598[b] |

[a] Values 400 and lower with either 500 or 550 ppt samples are false negative. Values 401 and greater with samples containing <500 ppt are "initial test" false-violative (positive results with samples containing non-actionable levels of aflatoxin M1).

[b] Reading results greater than the limit (400 ppt).

[c] Cochran outlier excluded from analysis.

[d] Cochran straggler not excluded from analysis.

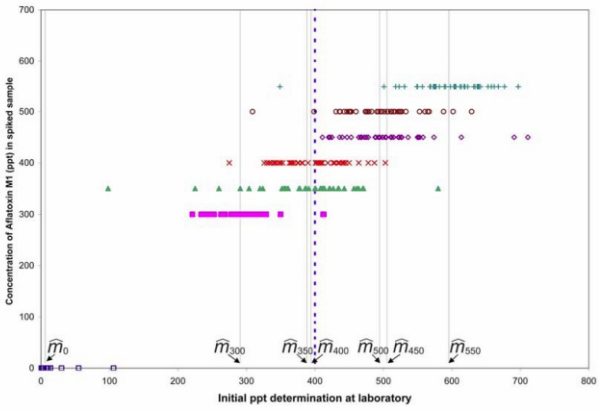


**Figure 1. Initial laboratory determinations of each added concentration and the calculated means. Negative (□), 300 ppt (■), 350 ppt (▲), 400 ppt (x), 450 ppt (◇), 500 ppt (¤), and 550 ppt (+) samples were plotted versus their spiked concentrations. Vertical lines $\hat{m}$0, 300, 350, 400, 450, 500, and 550 mark the mean determinations of the 21 laboratories for each of the fortified concentrations. Readings greater than the 400 ppt control point (▬ ▬) were initial test positive.**

550 ppt samples (10). All results at the 0 and 300 ppt were negative/non-actionable by the SLAFMQ method. At 350 ppt, the 9 false violatives represented 21% of the total, while at 400 ppt the 6 false violatives represented 14% of the samples. At 500 ppt, there were 3 of 42 (7%) false-negative/non-actionable results, and at 550 ppt there was 1 of 41 (2%) false-negative/non-actionable results.

The SLAFMQ method dose response, the initial test result dose response, and one data calculated 90% positive concentrations with 95% confidence are presented in Table 5. The control point at 400 ppt and retesting procedure was selected to provide a 90% positive concentration with 95% confidence at the 500 ppt action level while limiting false-violative results. The SLAFMQ method 90% positive concentration with 95% confidence was 503 ppt, which is a shift from 470 ppt calculated from the initial test result. The SLAFMQ method as compared to running only the initial test reduced the false-violative results at 350 ppt from 52 to 21% and at 400 ppt from 50 to 14%.

The false-violative rate from all samples at 400 ppt and less was 15 positives out of 167 samples, or 9%. The false-violative rate at 450 ppt was 93%. A high false-violative rate at 450 ppt was expected, as the SLAFMQ threshold level of 400 ppt was chosen to minimize false negatives. Acceptance of false-violative results from samples containing >400 but <500 ppt was consistent with a recent single laboratory evaluation of aflatoxin M1 tests (Trujillo et al., poster presented at the 2005 AOAC Annual Meeting in Orlando, FL).

The false-negative rate for 500 and 550 ppt samples was 4 negatives out of 83 results, or 4.8% of the samples. The false-negative rate for the SLAFMQ method was twice that of performing just the initial test where the false-negative rate was 2.4%. The false-negative rate of the initial test met criteria for single laboratory evaluations of NCIMS screening tests (12). Performance criteria for multiple laboratory testing have not been established by NCIMS. Multiple laboratory evaluations of methods typically follow AOAC and ISO/IDF guidelines and are more robust than single laboratory evaluations.

The HPLC results from 5 laboratories are reported in Table 6. There were no false-violative results in the final reported determinations. A 38% false-negative rate was found since 9 of 24 samples at 500 or 550 ppt aflatoxin M1 were reported to contain less than the 500 ppt action level. HPLC results indicated some interlaboratory variation with Laboratory A, showing close agreement to the prepared study standards. HPLC methods do not apply a threshold for variation at 500 ppt to achieve a 90% positive concentration with 95% confidence. Laboratories A, C, D, and E performed AOAC Method **2000.08** (5), which yielded more acceptable results than data from performing Method **986.16** (6) by Laboratory B. Overall, the HPLC determined concentrations of aflatoxin M1 in the samples were consistent with the prepared fortified aflatoxin M1 concentrations in milk.

The intralaboratory means at each concentration ($\hat{m}_0$–$\hat{m}_{550}$) from performing the SLAFMQ method are presented in Table 7 and Figure 1. The mean values from analysis of the 300, 400, and 500 ppt samples correlated within 3% of the prepared concentrations. The mean values from analysis of the 350, 450, and 550 ppt samples correlated within 14% and trended more positive than the prepared concentrations. This positive bias when using this raw milk to prepare samples containing 350, 450, and 550 ppt aflatoxin M1 may explain the positive trend in these samples and why the 450 ppt sample mean ($\hat{m}_{450}$ = 505 ppt) was greater than the 500 ppt sample mean ($\hat{m}_{500}$ = 495 ppt).

Intralaboratory repeatability and interlaboratory reproducibility of data from analysis of the blind sample pairs (Table 2) were calculated and are presented in Table 7. Repeatability is the range of determinations that can be expected from multiple analyses on the same sample in a single laboratory. Reproducibility is the range of determinations that could be expected from multiple analyses of identical samples in multiple laboratories. The $CV_r$% of repeatability ($RSD_r$) at each concentration was <16% and on average was 11% of the determined concentration. The $CV_R$% of reproducibility ($RSD_R$) at each concentration was <20% and on average was 13% of the determined concentration. The repeatability ($r$) and reproducibility ($R$) values represent 2.8 standard deviations of the laboratory determinations. The values at 550 ppt, of $r$ = 101 and $R$ = 126, were half of those determined by the HPLC method at 580 ppt, which were $r$ = 203 and $R$ = 310 (13). These statistical parameters represent the 95% variation range expected from identical sample determinations within a laboratory ($r$) and between different

**Table 3. Duplicate retests of initial positive samples[a]**

| ID | Added concn, ppt | Laboratory | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| 1 | 450 | 459[b] | 448[b] | 416[b] | 463[b] | 479[b] | 463[b] | 561[b] | 445[b] | 512[b] | 497[b] | 621[b] | 496[b] | 533[b] | 515[b] | 577[b] | 582[b] | 541[b] | 483[b] | 506[b] | 517[b] | 694[b] |
| | | 573[b] | 472[b] | 497[b] | 321 | 434[b] | 449[b] | 476[b] | 462[b] | 535[b] | 546[b] | 581[b] | 499[b] | 571[b] | 446[b] | 426 | 431[b] | 547[b] | 479[b] | 505[b] | 429[b] | 704[b] |
| 2 | 300 | | | | | | | | | | | | | | | | | | | | | 408[b] |
| | | | | | | | | | | | | | | | | | | | | | | 315 |
| 3 | 350 | | 393 | | | | 314 | 435[b] | 367 | | 358 | 447[b] | | 341 | 352 | | | | 391 | 434[b] | | 610[b] |
| | | | 301 | | | | 365 | 414[b] | 361 | | 449[b] | 469[b] | | 408[b] | 470[b] | | | | 384 | 451[b] | | 625[b] |
| 4 | 500 | 540[b] | 442[b] | 498[b] | 611[b] | 414[b] | 433[b] | 522[b] | 510[b] | 553[b] | 518[b] | 437[b] | 496[b] | 526[b] | 501[b] | [c] | 516[b] | 462[b] | 513[b] | 488[b] | 512[b] | 485[b] |
| | | 417[b] | 477[b] | 514[b] | 447[b] | 451[b] | 468[b] | 451[b] | 490[b] | 443[b] | 524[b] | 591[b] | 453[b] | 492[b] | 430[b] | [c] | 483[b] | 420[b] | 542[b] | 484[b] | 441[b] | 476[b] |
| 5 | 300 | | | | | | | | | | | | | | | | | | | | | 422[b] |
| | | | | | | | | | | | | | | | | | | | | | | 394 |
| 6 | 450 | 440[b] | 486[b] | 321 | 403[b] | 505[b] | 479[b] | 474[b] | 527[b] | 546[b] | 541[b] | 556[b] | 488[b] | 549[b] | 434[b] | 445[b] | 531[b] | 455[b] | 555[b] | 465[b] | 475[b] | 645[b] |
| | | 493[b] | 480[b] | 385 | 393 | 471[b] | 450[b] | 471[b] | 517[b] | 553[b] | 587[b] | 557[b] | 489[b] | 525[b] | 479[b] | 468[b] | 464[b] | 502[b] | 531[b] | 464[b] | 423[b] | 655[b] |
| 7 | 550 | 609[b] | 466[b] | 539[b] | 618[b] | 526[b] | 426[b] | 569[b] | 633[b] | 593[b] | 568[b] | 750[b] | 572[b] | 579[b] | 706[b] | 603[b] | 631[b] | 535[b] | 639[b] | [d] | 549[b] | 674[b] |
| | | 592[b] | 623[b] | 526[b] | 593[b] | 595[b] | 520[b] | 643[b] | 617[b] | 674[b] | 595[b] | 634[b] | 579[b] | 585[b] | 571[b] | 512[b] | 584[b] | 531[b] | 626[b] | | 610[b] | 730[b] |
| 8 | 400 | | 472[b] | 399 | 448[b] | | | | | 422[b] | | 432[b] | | 377 | 514[b] | | 429[b] | | | | | 528[b] |
| | | | 417[b] | 386 | 322 | | | | | 391 | | 358 | | 391 | 448[b] | | 351 | | | | | 526[b] |
| 9 | 0 | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | |
| 10 | 0 | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | |
| 11 | 350 | 414[b] | | 364 | | | | 414[b] | 438[b] | 414[b] | | 444[b] | | 583[b] | 400[b] | | 367 | 409[b] | 362 | | | 519[b] |
| | | 383 | | 361 | | | | 386 | 462[b] | 459[b] | | 459[b] | | 380 | 361 | | 340 | 429[b] | 394 | | | 427[b] |
| 12 | 500 | 457[b] | 494[b] | 457[b] | [c] | 440[b] | 418[b] | 485[b] | 532[b] | 425[b] | 453 | 485[b] | 483[b] | 585[b] | 608[b] | 496[b] | 514[b] | 410[b] | 481[b] | 501[b] | 459[be] | 630[b] |
| | | 499[b] | 486[b] | 466[b] | [c] | 480[b] | 419[b] | 491[b] | 562[b] | 479[b] | 526 | 503[b] | 523[b] | 501[b] | 488[b] | 448[b] | 489[b] | 420[b] | 419[b] | 519[b] | 381[e] | 585[b] |
| 13 | 400 | 404[b] | 328 | | | | | 400 | 386 | 393 | | 402[b] | 385 | 418[b] | 411[b] | | 397 | 388 | 365 | | | |
| | | 407[b] | 266 | | | | | 397 | 387 | 456[b] | | 363 | 393 | 421[b] | 415[b] | | 360 | 441[b] | 430[b] | | | |
| 14 | 550 | 731[b] | 398[e] | 561[b] | 551[b] | 521[b] | 644[b] | 575[b] | 589[b] | 605[b] | 575 | 553[b] | 519[b] | 623[b] | 546[b] | 632[b] | 589[b] | 510[b] | 625[b] | 688[b] | 527[b] | 587[b] |
| | | 750[b] | 329[e] | 614[b] | 438[b] | 573[b] | 579[b] | 536[b] | 580[b] | 537[b] | 578 | 548[b] | 542[b] | 637[b] | 601[b] | 603[b] | 570[b] | 596[b] | 494[b] | 570[b] | 610[b] | 688[b] |

[a] The 300, 350, 400, and 450 ppt samples testing >400 ppt on both retests were SLAFMQ false violatives.
[b] Reading results greater than the limit (400 ppt).
[c] Initial false negative; result not retested.
[d] Initial test result was an outlier and excluded.
[e] The 500 or 550 ppt samples that tested negative and were false negative.

laboratories ($R$). Lower $r$ and $R$ values and very low HorRat values, $HorRat_r < 0.3$ and $HorRat_R < 0.5$, indicate that the SLAFMQ method may have greater precision than HPLC for quantitation of aflatoxin M1 levels in milk.

## Conclusions

The SLAFMQ assay for raw milk in a 21 laboratory interlaboratory study detected U.S. and Codex action levels with a 90% positive concentration with 95% confidence at 503 ppt and a 4.8% false-negative rate. False violatives were minimized using a confirmation procedure that required samples with initial positive results to be retested twice, and for both retests to be positive. The calculated repeatability ($r$) and reproducibility ($R$) for the SLAFMQ method were lower than published values for HPLC methods at comparable concentrations. The SLAFMQ method had greater confidence at detecting actionable samples, at 500 and 550 ppt, than HPLC methods performed on the same samples.

**Table 4. Positive/actionable or negative/non-actionable determinations after retesting[a]**

| | | Laboratory | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | Added concn, ppt | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| 1 | 450 | + | + | + | – | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 2 | 300 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 3 | 350 | – | – | – | – | – | – | + | – | – | – | + | – | – | – | – | – | – | – | + | – | + |
| 4 | 500 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | + | + | + | + | + | + |
| 5 | 300 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 6 | 450 | + | + | – | – | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 7 | 550 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | [b] | + | + |
| 8 | 400 | – | + | – | – | – | – | – | – | – | – | – | – | – | + | – | – | – | – | – | – | + |
| 9 | 0 | – | – | – | – | – | – | – | – | – | – | – | – | – | [b] | – | – | – | – | – | – | – |
| 10 | 0 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 11 | 350 | – | – | – | – | – | – | – | + | + | – | + | – | – | – | – | – | + | – | – | – | + |
| 12 | 500 | + | + | + | – | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | + | + |
| 13 | 400 | + | – | – | – | – | – | – | – | – | – | – | – | + | + | – | – | – | – | – | – | – |
| 14 | 550 | + | – | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

[a] + = Positive/actionable results. – = Negative/non-actionable results. Samples at 500 and 550 ppt that tested negative were false negatives. The 300, 350, 400, and 450 ppt samples testing positive were false violatives.

[b] Initial test result was an outlier and excluded.

**Table 5. Dose responses of SLAFMQ method and initial test with 90% positive concentrations with 95% confidence**

| | | SLAFMQ method | | Initial test | |
|---|---|---|---|---|---|
| Added concn, ppt | No. of samples tested | No. positive | % Positive | No. positive | % Positive |
| 0 | 41[a] | 0 | 0 | 0 | 0 |
| 300 | 42 | 0 | 0 | 2 | 5 |
| 350 | 42 | 9 | 21 | 22 | 52 |
| 400 | 42 | 6 | 14 | 21 | 50 |
| 450 | 42 | 39 | 93 | 42 | 100 |
| 500 | 42 | 39 | 93 | 40 | 95 |
| 550 | 41[a] | 40 | 98 | 41 | 100 |
| 90% Positive concentration with 95% confidence, ppt | | 503 | | 470 | |
| Pearson Chi square | | 25.7 | | 20.2 | |

[a] Outliers at 0 and 550 removed.

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**Table 6. HPLC determinations of samples reported in ppt[a]**

| Sample No. | Added concn, ppt | HPLC results reported by labs | | | | | | HPLC raw data of Lab C (run 1) and Lab B (runs 1–3) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A[b] | B[c] | C 1[d] | C 2[d] | D[e] | E[e] | C 1 reported[d] | B (1st run)[c] | B (2nd run)[c] | B (3rd run)[c] |
| 15 | 450 | 430 | 380 | 442 | 246 | 490 | 375 | 602[f] | 330 | 240 | 560[f] |
| 16 | 550 | 530[f] | 520[f] | 598[f] | 457[g] | 460[g] | 476[g] | 814 | 450[g] | 270[g] | 850[f] |
| 17 | 450 | 470 | 480 | 355 | 341 | 420 | 439 | 483 | 370 | 230 | 370 |
| 18 | 500 | 550[f] | 500[f] | 584[f] | 363[g] | 470[g] | 514[f] | 794[f] | 460[g] | 310[g] | 720[f] |
| 19 | 400 | 450 | 310 | 389 | 299 | 440 | 343 | 529[f] | 310 | 310 | |
| 20 | 300 | 370 | 220 | 343 | 293 | 300 | 292 | 467 | 220 | 220 | |
| 21 | 350 | 380 | 260 | 437 | 306 | 370 | 306 | 595[f] | 260 | 260 | |
| 22 | 400 | 350 | 340 | 302 | 140 | 390 | 337 | 411 | 340 | 340 | |
| 23 | 500 | 500[f] | 400[g] | 474[g] | 307[g] | 520[f] | 418[g] | 645[f] | 110[g] | 380[g] | 710[f] |
| 24 | 350 | 320 | 350 | 357 | 304 | 260 | 295 | 485 | 160 | 140 | 460 |
| 25 | 300 | 280 | 260 | 378 | 240 | 290 | 292 | 514[f] | 160 | 160 | 440 |
| 26 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 27 | 550 | 500[f] | 540[f] | 609[f] | 535[f] | 560[f] | 559[f] | 828[f] | 340[g] | 510[f] | 760[f] |
| 28 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |

[a] HPLC values reported by laboratories were in ppb and were converted to ppt. The 450, 400, 350, and 300 ppt samples testing >500 ppt were false-violative results.

[b] Laboratory A performed AOAC Method **2000.08**, Affinity purification of aflatoxin M1 followed by derivatization and HPLC-fluorescence detection.

[c] Laboratory B performed (HPLC) AOAC Method **986.16** and reported possible problems with injector apparatus and requested average of 3 determinations be reported. Raw data are displayed as 1st, 2nd, and 3rd run (last 3 columns).

[d] Laboratory C performed (HPLC) AOAC Method **2000.08** and initially reported values "C 1 reported." When asked to investigate a positive bias, Laboratory C found a concentration problem with the calibration standard and corrected the original data to "C 1." The laboratory also retested the samples 1 week later after applying a correct standard, results "C 2."

[e] Laboratories D and E performed AOAC Method **2000.08** modified for reduced volume sample and tested samples 1–14 that were converted to the HPLC sample blind codes, 15–28.

[f] Results greater than the action level (500 ppt); true positives.

[g] The 500 or 550 ppt samples that tested lower than the action level and were false negatives.

**Table 7. Charm SLAFMQ results showing mean, repeatability, reproducibility, and HorRat statistics from data in Table 2**

| Added concn, ppt | Mean ($\hat{m}$) of SLAFMQ determinations | Intralaboratory repeatability statistics | | | | Interlaboratory reproducibility statistics | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $StDev_r$ | $CV_r$% ($RSV_r$) | Repeatability $r = 2.8\ (StDev_r)$ | $HorRat_r$ value ($RSV_r$/$PRSV_R$) | $SD_R$ | $CV_R$% ($RSV_R$) | Reproducibility $R = 2.8\ (SDR_R)$ | $HorRat_R$ value ($RSV_R$/$PRSV_R$) |
| 0 | 6 | 6 | 119 | 16 | 1.25 | 13 | 262 | 36 | 2.73 |
| 300 | 291 | 31 | 11 | 86 | 0.20 | 41 | 14 | 115 | 0.26 |
| 350 | 388 | 61 | 16 | 170 | 0.30 | 78 | 20 | 218 | 0.39 |
| 400 | 394 | 40 | 10 | 112 | 0.20 | 50 | 13 | 139 | 0.25 |
| 450 | 505 | 48 | 9 | 133 | 0.19 | 63 | 12 | 176 | 0.25 |
| 500 | 495 | 62 | 13 | 173 | 0.25 | 62 | 13 | 173 | 0.25 |
| 550 | 596 | 36 | 6 | 101 | 0.12 | 45 | 8 | 126 | 0.15 |

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