Immunofa\textsuperscript{a}inity Column Cleanup with Liquid Chromatography for Determination of Aflatoxin M\textsubscript{1} in Liquid Milk: Collaborative Study

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A collaborative study was conducted to evaluate the effectiveness of an immunofa\textsuperscript{a}inity column cleanup liquid chromatographic method for determination of aflatoxin M\textsubscript{1} in milk at proposed European regulatory limits. The test portion of liquid milk was centrifuged, filtered, and applied to an immunofa\textsuperscript{a}inity column. The column was washed with water, and aflatoxin was eluted with pure acetonitrile. Aflatoxin M\textsubscript{1} was separated by re\textsuperscript{v}ersed-phase liquid chromatography (LC) with fluorescence detection. Frozen liquid milk samples both naturally contaminated with aflatoxin M\textsubscript{1} and blank samples for spiking, were sent to 12 collaborators in 12 different European countries. Test portions of samples were spiked at 0.05 ng aflatoxin M\textsubscript{1} per mL. After removal of 2 noncompliant sets of results, the mean recovery of aflatoxin M\textsubscript{1} was 74\%. Based on results for spiked samples (blind pairs at 1 level) and naturally contaminated samples (blind pairs at 3 levels) the relative standard deviation for repeatability (RSD\textsubscript{r}) ranged from 8 to 18\%. The relative standard deviation for reproducibility (RSD\textsubscript{p}) ranged from 21 to 31\%. The method showed acceptable within- and between-laboratory precision data for liquid milk, as evidenced by HORRAT values at the low level of aflatoxin M\textsubscript{1} contamination.

Methodology for determination of aflatoxin M\textsubscript{1} in milk improved markedly with the application of immunofa\textsuperscript{a}inity column technology to provide a combined extraction and cleanup stage to the analysis (1). Previously, methods involved either liquid–liquid extraction (2, 3) or solid phase extraction (4) followed by silica gel column or other cleanup (3, 5) with thin layer chromatography or liquid chromatographic (LC) determination. These critically compared methods (6) have formed the basis of full collaborative studies and are still extant as AOAC INTERNATIONAL Off\textsuperscript{i}cial Methods for aflatoxin M\textsubscript{1} in liquid and powdered milk (7). Although an immunofa\textsuperscript{a}inity LC method for determining aflatoxin M\textsubscript{1} in milk powder was collaboratively tested under the auspices of the International Dairy Federation (IDF; 8), the study lacked samples to establish method recovery and was not submitted to AOAC INTERNATIONAL for proposed adoption.

European Commission Regulations (9) for aflatoxin M\textsubscript{1}, implemented in January 1999, set a limit of 0.05 ng/mL in liquid milk. The existing AOAC method (10) has not been tested at <0.08 ng/mL, and the IDF method (8) has only been tested for powdered milk at a 10-fold lower limit when expressed on an equivalent weight basis. As part of a project funded by the European Commission Standards Measurement and Testing (SMT) Programme on method validation, a full collaborative study was undertaken at the low European limit required by the new regulations. This validated method will ultimately be submitted for consideration for adoption as a European Standard (CEN), and will be aimed at fulfilling AOAC INTERNATIONAL requirements for a collaborative study.

Because contamination levels involved in the present study were very low, particular care was taken in the preparation, homogeneity testing, packaging, and storage of liquid milk test samples. All laboratories were provided with a common standard of aflatoxin M\textsubscript{1}, the concentration of which was confirmed by 3 independent laboratories at the outset of the trial. To ensure that all collaborative trial participants rigorously followed the protocol, a precollaborative trial workshop was held in January 1998. The workshop did not involve any hands-on
analytical work, but did provide opportunities to discuss and raise any potential difficulties before the start of the trial.

**Collaborative Study**

**Test Materials**

**Preparation of milk samples.**—Naturally contaminated milk was prepared by feeding cows with aflatoxin B<sub>1</sub>-contaminated peanut meal. Two cows were fed with 2 kg contaminated peanut meal (containing about 2 mg/kg aflatoxin B<sub>1</sub>) added to their daily ration for 3 days. The morning milkings on the fourth day (about 11 L milk) were collected and gently homogenized to keep the fat well dispersed. Analysis of the aflatoxin M<sub>1</sub> content of this milk indicated a level of contamination of 2.2 ng/mL. The milk was stored at −30°C until use.

Control milk (30 L) containing <0.005 ng/mL aflatoxin M<sub>1</sub>, determined by the method described in this study, was obtained from a local farm. The milk was stabilized with penicillin at about 0.06 µg/mL, and divided into 12 L for use as the control sample for the study and 18 L for use in blending to generate the naturally contaminated samples.

**Preparation of test materials to be sent to laboratories.**—Different volumes (68.5, 137, and 274 mL) of the highly contaminated milk (containing 2.2 ng/mL aflatoxin M<sub>1</sub>) were diluted with 6 L blank milk in each case. After thorough mixing, the milk was subdivided into 125 mL Nalgene plastic bottles (56 samples in each case). All samples were frozen at −30°C.

**Homogeneity testing of milk samples.**—Every sixth sample of contaminated milk and every twelfth sample of blank milk were removed from each batch, providing 10 samples of milk and every twelfth sample of blank milk. Each sample was analyzed by the method described here in duplicate for aflatoxin M<sub>1</sub> content.

**Organization of the Collaborative Study**

The 12 collaborators from 12 different European countries represented a cross-section of government, food control, university, and food industry affiliations. Before the trial, each collaborator received a practice sample of blank milk and a calibrant solution for spiking. Collaborators met at a precollaborative trial workshop where any problems experienced with analyzing the practice sample were discussed, and details of the organization of the trial were outlined by the coordinators.

For the collaborative trial, each participant received the following: (1) a set of 8 randomly coded samples of liquid milk; (2) a pair of blank milk samples for spiking; (3) one labeled ampule of aflatoxin M<sub>1</sub> calibrant solution provided by the European Commission, SMT Programme, with an independently established aflatoxin M<sub>1</sub> content of 10 µg/mL; (4) 2 ampules of aflatoxin M<sub>1</sub> calibrant solution labeled A and B, with aflatoxin M<sub>1</sub> content unknown to participants; (5) 10 immunoaffinity columns containing anti-aflatoxin M<sub>1</sub> antibodies, which were supplied from the same batch; (6) a copy of the method of analysis; and (7) instructions for undertaking the collaborative study.

Frozen milk samples, together with ice-packs, were sent to the laboratories by express delivery. Each participant was required to prepare one extract from each milk sample and analyze by LC. Participants were also provided with a spiking protocol and 2 bottles of milk blank materials assumed to contain <0.005 aflatoxin M<sub>1</sub> ng/mL. Participants were asked to spike blank materials by opening aflatoxin M<sub>1</sub> ampule A, transfer 50 µL of the calibration solution into a vial, evaporate it to dryness under a gentle stream of nitrogen, and add 1 mL 10% acetonitrile solution. After labeling this solution “vial 1,” participants were to agitate it vigorously with a vortex-like stirrer. Then, participants were to transfer 50 µL from vial 1 to 950 µL 10% acetonitrile solution, label this solution as “vial 2,” and shake it vigorously with a vortex-like stirrer. Participants were to measure 70 mL of blank milk and transfer 1 mL milk from the 70 mL volume of blank material to a 2 or 2.5 mL tube, and add 140 µL from “vial 2.” After shaking vigorously with a Vortex-like stirrer for about 30 s, participants were to dilute this spiked solution in the remaining volume (original volume less 1 mL) of blank material, and shake it again vigorously for a further 30 s. Participants were to analyze this spiked material by following exactly the procedure given in the method protocol, taking a test portion of 50 mL. The whole spiking sequence was repeated with the second bottle of blank material and the aflatoxin M<sub>1</sub> ampule B. This spiking protocol led to a spiking level of 0.050 ng/mL.

**AOAC Official Method 2000.08**

*Aflatoxin M<sub>1</sub> in Liquid Milk*

**Imunoaffinity Column by Liquid Chromatography**

*First Action 2000*

(Applicable to determination of aflatoxin M<sub>1</sub> in raw liquid milk at > 0.02 ng/mL).

**Caution:** This method requires the use of solutions of aflatoxin M<sub>1</sub>. Aflatoxins are carcinogenic to humans. See introductory statement to this chapter (11). Aflatoxins are subject to light degradation. Protect analytical work from the daylight and keep aflatoxin standard solutions protected from light by using amber vials or aluminum foil. The use of non acid-washed glassware (e.g., vials, tubes, flasks) for aflatoxin aqueous solutions may cause a loss of aflatoxin. Special attention should be taken with new glassware. Thus, before use, soak glassware in dilute acid (e.g., sulfuric acid, 110 mL/L) for several hours; then, rinse extensively with distilled water to remove all traces of acid (check with pH paper).

See Table 2000.08A for the results of the interlaboratory study supporting acceptance of the method.

**A. Principle**

The test portion is extracted and cleaned up by passing through an immunoaffinity column containing specific antibodies bound onto a solid support. Antibodies selectively bind...
with any aflatoxin $M_1$ (antigen) contained in the extract, to give an antibody–antigen complex. Other components of matrix are washed off the column with water. Aflatoxin $M_1$ from the column is eluted with acetonitrile. After the eluate is concentrated, the amount of aflatoxin $M_1$ is determined by LC with fluorometric detection.

**B. Performance Standards for Immunoaffinity Columns**

The immunoaffinity column shall contain antibodies against aflatoxin $M_1$ with a capacity of not less than 100 ng aflatoxin $M_1$ (which corresponds to 2 ng/mL when 50 mL test portion is applied). Recovery of not less than 80% must be obtained for aflatoxin $M_1$ when a calibrated solution containing 4 ng toxin is applied (which corresponds to 80 ng/L for a loaded volume of 50 mL).

Any immunoaffinity column meeting the above specifications can be used. Check the performance of the columns regularly, at least once for every batch of columns.

**C. Apparatus**

1. **Disposable syringe barrels.**—To be used as reservoirs (10 and 50 mL capacity).
2. **Vacuum system.**—For use with immunoassay columns.
3. **Centrifuge.**—To produce a radial acceleration of at least 2000 × g.
4. **Micropipettes.**
5. **Microsyringes.**—100, 250, and 500 µL (Hamilton or equivalent).
6. **Glass beakers.**
7. **Volumetric flasks.**—50 mL.
8. **Water bath.**—37 ± 2°C.
9. **Filter paper.**—Whatman No. 4, or equivalent.
10. **Conical glass tubes.**—5 and 10 mL, stoppered.
11. **Spectrophotometer.**—Wavelength 200–400 nm, with quartz face cells of optical length 1 cm.
12. **Vacuum system.**

**C. Solution is stable ca 1 year.**

**D. Reagents**

1. **Chloroform.**—Stabilized with 0.5–1.0% ethanol.
2. **Nitrogen.**
3. **Aflatoxin $M_1$ standard solutions.**—(1) **Stock standard solution.**—1 µg/mL. Suspend a lyophilized film of reference standard aflatoxin $M_1$ in chloroform to obtain the required concentration. Determine the concentration of aflatoxin $M_1$ by measuring its absorbance at the maximum (ca 365 nm) in a calibrated spectrophotometer against chloroform as a blank between 200–400 nm. Check purity by noting an undistorted shape of the recorded peak. Calculate the mass concentration ($C$, µg/mL) from the equation:

$$ C = \frac{1000AM}{\varepsilon} $$

where $A$ is the measured absorbance at the maximum wavelength, $M$ is the molecular mass of aflatoxin $M_1$ (328 g/mol), and $\varepsilon$ is the absorption coefficient of aflatoxin $M_1$ in chloroform (1995 m²/mol; 12, 13).

Store this stock solution in a tightly stoppered amber vial below 4°C. Solution is stable ca 1 year.

(2) **Working standard solution.**—1 µg/mL. Transfer by means of a syringe 50 µL of the standard stock solution, (c)/(1), into an amber vial and evaporate to dryness under a steady stream of N. Dissolve the residue vigorously in 500 µL acetonitrile using a Vortex mixer. Store this solution in a

**Table 2000.08A. Interlaboratory study results for aflatoxin $M_1$ in liquid milk immunoaffinity column LC method**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>No. of labs, a(b)$^a$</th>
<th>$\bar{x}$ Average (ng/mL)</th>
<th>$r$</th>
<th>$s_r$</th>
<th>RSD, %</th>
<th>$R$</th>
<th>$s_R$</th>
<th>RSDr, %</th>
<th>HORRAT value</th>
<th>Rec., %</th>
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<tbody>
<tr>
<td>Spiked</td>
<td>10 (2)</td>
<td>0.037$^b$</td>
<td>0.019</td>
<td>0.007</td>
<td>18</td>
<td>0.032</td>
<td>0.011</td>
<td>31</td>
<td>0.42</td>
<td>74</td>
</tr>
<tr>
<td>a</td>
<td>12 (0)</td>
<td>&lt;0.005</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>12 (0)</td>
<td>0.023</td>
<td>0.011</td>
<td>0.004</td>
<td>17</td>
<td>0.017</td>
<td>0.006</td>
<td>27</td>
<td>0.33</td>
<td>93</td>
</tr>
<tr>
<td>c</td>
<td>12 (0)</td>
<td>0.046</td>
<td>0.016</td>
<td>0.006</td>
<td>12</td>
<td>0.029</td>
<td>0.010</td>
<td>23</td>
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<td>94</td>
</tr>
<tr>
<td>d</td>
<td>12 (0)</td>
<td>0.103</td>
<td>0.022</td>
<td>0.008</td>
<td>8</td>
<td>0.062</td>
<td>0.022</td>
<td>21</td>
<td>0.33</td>
<td>107</td>
</tr>
</tbody>
</table>

$^a$ Number of labs retained after eliminating outliers; $^b$ number of labs removed as outliers.

Note: Statistical analysis was not carried out on the blank milk (a).
tightly stoppered amber vial below 4°C. Solution is stable ca 1 month.

(3) Calibration standard solutions.—Prepare on day of use. Bring working standard solution, (c)(2), to ambient temperature. Prepare a series of standard solutions in the mobile phase, C(n), of concentrations that depend upon the volume of the injection loop in order to inject, e.g., 0.05–1.0 ng aflatoxin M₁.

E. Preparation of Test Solution

Warm milk before analysis to ca 37°C in a water bath, and then gently stir with magnetic stirrer to disperse the fat layer. Centrifuge liquid milk at 2000 × g to separate the fat and discard thin upper fat layer. Filter through one or more paper filters, collecting at least 50 mL. Let immunoaffinity columns reach room temperature. Attach syringe barrel to top of immunoaffinity cartridge. Transfer 50 mL (Vₛ) of prepared test portion with volumetric flask volumetric pipet into syringe barrel and let it pass through immunoaffinity column at slow steady flow rate of ca 2–3 mL/min. Gravity or vacuum system can be used to control flow rate.

Remove syringe barrel and replace with a clean one. Wash column with 20 mL water at steady flow rate. After washing completely, blow column to dryness with N stream. Put another dry clean barrel on the cartridge. Slowly elute aflatoxin M₁ from column with 4 mL pure acetonitrile. Allow acetonitrile to be in contact with column at least 60 s. Keep steady slow flow rate. Collect eluate in conical tube. Evaporate eluate to dryness using gentle stream of N. Dilute to volume Vₕ of mobile phase, i.e., 200 µL (for 50 µL injections) to 1000 µL (for 250 µL injections).

F. LC Determination with Fluorescence Detection

Pump mobile phase at steady flow rate through LC column. Depending on the kind of column, the acetonitrile–water ratio and flow rate of the mobile phase may be adjusted to ensure optimal separation of aflatoxin M₁ from other extract components. As a guideline for conventional columns (with a length of 250 mm and id of 4.6 mm), a flow rate of ca 0.8 mL/min gives optimal results. Check optimal conditions with aflatoxin M₁ calibrant solution and spiked milk before analyzing test materials.

Check linearity of injection calibrant solutions and stability of chromatographic system. Repeatedly inject a fixed amount of aflatoxin M₁ calibrant solution until stable peak areas or heights are obtained. Peak areas or heights corresponding to consecutive injections must be within ± 5%. Retention times of aflatoxin M₁ can vary as a function of temperature and must be monitored by injecting a fixed amount of aflatoxin M₁ calibrant solution at regular intervals.

(1) Calibration curve of aflatoxin M₁.—Inject in sequence suitable volumes Vᵢ, depending on the injection loop, aflatoxin M₁ standard solutions containing from 0.05 to 1 ng. Prepare a calibration graph by plotting the peak area or peak height against the mass of injected aflatoxin M₁.

(2) Analysis of purified extracts and injection scheme.—Inject suitable volume Vᵢ (equivalent to at least 12.5 mL milk) of eluate into LC apparatus through injection loop. Using the same conditions as for calibrant solutions, inject calibrants and test extracts according to stipulated injection scheme. Inject an aflatoxin M₁ calibrant with every 10 injections. Determine aflatoxin M₁ peak area or height corresponding to the analyte, and calculate aflatoxin M₁ amount Wₐ in test material from the calibration graph, in ng. If aflatoxin M₁ peak area or height corresponding to test material is greater than the highest calibrant solution, dilute the eluate quantitatively with mobile phase and re-inject the diluted extract into the LC apparatus.

G. Calculation

Calculate aflatoxin M₁ mass concentration of the test sample, using the following equation:

\[ W_m = W_a \times \left( \frac{V_f}{V_i} \right) \times \left( \frac{1}{V_s} \right) \]

where \( W_m \) = the numerical value of aflatoxin M₁ in the test sample in ng/mL (or µg/L); \( W_a \) = the numerical value of the amount of aflatoxin M₁ corresponding to area or height of the aflatoxin M₁ peak of the sample extract (ng); \( V_f \) = the numerical value of the final volume of redissolved eluate (µL); \( V_i \) = the numerical value of the volume of injected eluate (µL); \( V_s \) = the numerical value of volume of prepared test portion passing through the column (mL).

Express the results to 3 significant figures.
Ref.: J. AOAC Int. 84, 438–440(2001)

Results and Discussion

Homogeneity of Test Materials

The replicate analysis of every sixth sample of milk from each batch indicated that at all 3 levels, the contaminated milk samples were homogeneous. No trend was observed for either sampling or analysis order for all samples, thus, confirming overall that the samples were homogeneous.

Precollaborative Trial Workshop

Only minor points to clarify details of the method were requested at the workshop. This resulted in a closer definition of the centrifugation conditions (>2000 g but <4000 g) and optimization of the acetonitrile–water ratio for the LC mobile phase.

Collaborative Trial

Participants 3 and 9 reported receiving milk samples that were curdled upon arrival or became curdled after storage. Participants 3 and 9 were sent a second set of samples.

The method protocol allowed a choice of LC column for the analysis, and information was collected on the instrumentation used. A diversity of LC columns (type, dimension, manufacturer) were used by participants. Many participants chose to use short columns (100 × 4 or 5) to reduce the amount of solvents used. Most participants used the recommended LC mobile phase (water–acetonitrile, 75 + 25) but 2 participants selected a ternary mobile phase (water–acetonitrile–methanol, 65 + 25 + 10) for an ODS-1 column and (water–isopropanol–acetonitrile, 80 + 12 + 8) for an ODS
Hypersil column but without indication of evident advantage. The flow rate for delivery of mobile phase in the LC apparatus varied according to the length of the LC columns.

The cleanup step was carried out manually or with the help of a vacuum system such as the VacElut™ system. No participant chose to use an automated system such as the ASPEC™. However, only one participant used a manual injection system (participant 3). The injection volumes ranged from 10 to 500 μL. No particular analytical effects were observed in relation to this wide discrepancy in the equipment of laboratories, which may be taken as tangible proof of the ruggedness of the method.

Comments from Collaborative Trial Participants

Some comments were made on the reporting sheets from participants. Laboratory 4 observed the possibility that an earlier-eluting peak corresponded to the occurrence of aflatoxin M₂ in the naturally contaminated milk. Irrespective of the identity of this earlier-eluting peak, it did not interfere with the aflatoxin M₁ peak and, therefore, did not hinder its accurate measurement. Laboratory 6 found the recommended acid washing and water rinsing of vials to be problematic. Laboratory 8 reported a different aflatoxin M₁ concentration in calibrant solution (25 μg/mL instead of 10 μg/mL). Laboratory 9 found it better to centrifuge the liquid milk at low temperature, and as with Laboratory 4, detected the presence of aflatoxin M₂ in all positive samples. Laboratory 11 observed that it would be easier to work with a test portion of 40 mL rather than the recommended 50 mL for extracting samples. Finally, except for Laboratory 6 which found that the method protocol was not clear enough with respect to the calculation equation, all other participants had no particular remarks concerning the understanding of the method protocol.

Spiking Experiment for Determining Recovery Yield of the Method

For determining the method recovery, laboratories were asked to undertake the spiking experiment. Laboratory raw data are reported in Table 1. Results from Laboratories 2 and 8 were removed as noncompliant as they had not adequately followed the spiking protocol. The running of Cochran and Grubbs tests did not identify any outliers. For the spiked samples, a repeatability RSDᵣ = 18%, and a reproducibility RSDᵣ = 31% were obtained for a mean overall recovery of 74%. Thus, notwithstanding the evident problems with recovery in the case of 3 participants (41, 45, and 51%), the performance characteristics for the spiked samples are still acceptable as confirmed by the HORRAT value of 0.42.

Although outside the scope of the statistical evaluation of collaborative trial data according to the International Harmonized Protocol (14), it was thought worthwhile to examine the influence of recovery on method performance. The data were, thus, reanalyzed after removing individual laboratory results where individual recovery was below an arbitrarily chosen 70%, i.e., removing 5 data sets from Laboratories 2, 3, 5, 6, and 8. This approach to data handling was previously used in consideration of laboratory intercomparison data for BCR, M&T (Measurement and Testing), and SMT certification exercises of reference materials, with 70% chosen as a minimum acceptable recovery. Removal of these data sets increased the mean percentage recovery to 87% and generated significantly better RSDᵣ and RSDᵣ values of 14% in both instances, with a HORRAT value of 0.19. The poor score for the 5 laboratories exhibiting a recovery <70 % was clearly related to mishandling in the spiking experiment or in the filtration step, as the milk used in this trial was raw milk, and not an indication of the recovery performance of the method itself.

Table 1. Collaborative trial results of determination of aflatoxin M₁ in liquid milk by LC

<table>
<thead>
<tr>
<th>Lab ID</th>
<th>0.05</th>
<th>0.05</th>
<th>a²</th>
<th>a</th>
<th>b²</th>
<th>b</th>
<th>c²</th>
<th>c</th>
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<td>&lt;0.0005</td>
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<td>&lt;0.002</td>
<td>0.027</td>
<td>0.030</td>
<td>0.051</td>
<td>0.058</td>
<td>0.120</td>
<td>0.121</td>
</tr>
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a, b, c, d = blind duplicate pairs of naturally contaminated samples.
b Noncompliant data (failure to correctly follow spiking procedure).
tories producing poor recoveries declared they had trouble in the filtration step (Laboratories 2, 3, 5, and 6) and Laboratories 5 and 6 had trouble filtering their spiked solutions. Laboratory 6 noted a slight flocculation of milk when dissolving the spiking solution. Laboratory 2 had diluted the calibrant solution in a too large volume of milk (30 mL) instead of the recommended volume (1 mL). Laboratory 8 did not evaporate the chloroform of the standard test portion for the spiking experiment which led to a lack of proper dissolution of aflatoxin M₁ calibrant solution in milk. It is also noteworthy that the LC injection volume for Laboratory 6 was quite small (10 μL) and for Laboratory 8 quite large (500 μL). This could possibly lead to a less accurate estimation in the measurement of the aflatoxin M₁ peak.

**Precision Characteristics of Method**

Raw data obtained from the interlaboratory study are given in full in (Table 1) and were not corrected for recovery. For the blank milks (sample ‘a’), all data with one exception (Laboratory 3 for one sample) were <0.005 μg/L which is unanimously considered as confirmation of the limit of quantification of the method at the signal-to-noise ratio of 5:1. This demonstrated that in no instances were any problems of interferences or co-extractives evident in the analysis of the milk extracts. The statistical evaluation was performed on uncorrected data according to the IUPAC/AOAC International Harmonized Protocol (14). The mean levels, precision parameters, and HORRAT values are given in Table 2000.08A. No straggling nor outlying data were found. The precision parameters are acceptable when considering the very low studied level of aflatoxin M₁ detection (i.e., below the μg/L level). The RSDᵣ is <31%. The acceptability of the precision values is confirmed by the very low HORRAT values (0.31–0.42) produced in this trial. There was no evidence of a significant overall improvement in precision data through selecting laboratories on the basis of recoveries above 70%, and subsequent recovery correction of the data.

Comparing results from this interlaboratory exercise to those already published on the validation of an LC fluorescence detection method for aflatoxin M₁ in liquid milk, the interlaboratory precision and HORRAT values are very similar for the same range of aflatoxin M₁ levels (i.e., roughly between 0.03 and 0.60 μg/L). In the present trial, RSDᵣ values of 21–27% are of the same order of magnitude as those of Tuinstra et al. (11–19%; 8), and better than those given in AOAC Method 986.16 (37–62%; 10), and the RSDᵣ of 28% obtained by Dragacci and Fremy (15) in proficiency testing where all participants used a very similar protocol.

**Recommendation**

It is recommended that the immunoaffinity column cleanup method by reversed-phase LC analysis with fluorescence detection be adopted Official First Action for determination of aflatoxin M₁ in liquid milk at >0.02 ng/mL.

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