Determination of Aflatoxins in Animal Tissues

ROBERT D. STUBBLEFIELD and ODETTE L. SHOTWELL
U.S. Department of Agriculture, Science and Education Administration, Northern Regional Research Center, Agricultural Research, Peoria, IL 61604

A method for the determination of aflatoxins in animal tissues has been developed, and applied successfully to beef, swine, chicken, and human livers, and to beef kidney, heart, spleen, muscle, and blood. Blended tissue is denatured with citric acid and extracted with dichloromethane on a wrist-action shaker. After filtration, the extract is partially purified on a silica gel column, and aflatoxins B1 and M1 are determined by 2-dimensional thin layer chromatography and densitometry. Recoveries of B1 and M1 added to meats and blood were approximately 90 and 80%, respectively. The method gave results for a contaminated freeze-dried liver comparable to analyses by 3 other published meat tissue methods. The method is rapid and has a determination limit ≤0.1 ng/g. In addition, the method uses less toxic and smaller quantities of solvents and chemicals.

Aflatoxins have been found in organs and tissues of beef, swine, and poultry that have ingested aflatoxin-contaminated feeds (1-3). The liver is the target organ of aflatoxicosis; however, B1, B2, and M1 have been detected in other edible animal tissues and, therefore, present a potential health hazard for humans. Aflatoxin levels reported in meats are usually less than 1 ng/g, but animal tissues contaminated with aflatoxin at any detectable level should be diverted from commercial food channels. This requires accurate and sensitive quantitative methods.

Several methods for determining aflatoxins in liver have been published (4-9). These methods either require considerable time, lack the desired detection limit ≤0.1 ng/g, or give final extracts for thin layer chromatography (TLC) that contain fluorescent impurities which make identification of low levels of aflatoxin difficult. A method for the determination of aflatoxin M1 in dairy products (10) was modified for animal tissues. The method is rapid and sensitive, and gives TLC extracts that are free of interfering contaminants. In addition, the method uses less toxic and smaller quantities of solvents and chemicals. This paper reports the application of this method to beef, swine, chicken, and human livers and to beef kidney, heart, spleen, muscle, and blood.

Experimental

Apparatus

(a) Wrist-action shaker.—Burrell, or equivalent.
(b) Meat grinder.—Waring Blender, Model EP-1, and any manual food grinder.
(c) Chromatographic columns.—Glass 50 × 1.0 cm id; see 26.A10(b) (11).
(d) Filter paper.—32 cm, S&S No. 588, or equivalent rapid flow, high wet-strength paper; and 24 cm, S&S No. 560, or Whatman 2V or equivalent medium flow paper.
(e) Thin layer plates.—10 × 10 cm commercial pre-poured, 0.25 mm thickness, glass plates (handcut from 20 × 20 cm) (E. Merck silica gel 60, No. 5763) or prepare in laboratory as described in 26.A10(d) (11).

Reagents

(a) Solvents.—Reagent grade, distilled in glass. Glacial acetic acid, acetone, acetonitrile, benzene, chloroform (0.75% ethanol), dichloromethane, ethyl ether (0.01% ethanol, peroxide-free), hexane (68–69°C), isopropanol, and toluene.
(b) Citric acid (20%).—Dissolve 200 g ACS grade citric acid monohydrate in 1 L water.
(c) Silica gel for column chromatography.—E. Merck silica gel 60 (No. 7734) 0.063–0.200 mm (70–230 mesh), or equivalent. Treat as described in 26.A11(c) (11).
(d) Sodium sulfate.—Anhydrous, granular.
(e) Diatomaceous earth.—Hyflo Super-Cel.
(f) Aflatoxin standards.—Prepare aflatoxins B1 and M1 in acetonitrile-benzene (1 + 9) to contain 0.25 μg/mL each for either visual or densitometric analysis. If aflatoxins G1, B2, and/or G2 are needed, prepare G1 at 0.25 μg/mL and B2 and G2 at 0.05 μg/mL. Store standards in 1 dram vials fitted with Teflon-lined screw caps and store at 0°F when not in use.

Samples

Artificially contaminated animal tissues.—All animal tissues and blood samples used in this...
Table 1. Recovery of aflatoxins B\(_1\) and M\(_1\) from artificially contaminated meat products

<table>
<thead>
<tr>
<th>Meat product</th>
<th>B(_1) and M(_1) added, ng/g or ng/mL</th>
<th>Aflatoxin B(_1)</th>
<th>Aflatoxin M(_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recd. ng/g or Recd. ng/mL, % or Std dev.,</td>
<td>Coeff. of var., %</td>
<td>Recd. ng/mL, % or Std dev.,</td>
</tr>
<tr>
<td>Liver, beef</td>
<td>0.1</td>
<td>0.091 90.7 0.013 14.6</td>
<td>0.084 84.4 0.013 15.4</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.476 95.2 0.031 6.6</td>
<td>0.409 81.1 0.036 8.9</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.922 92.2 0.132 14.3</td>
<td>0.817 81.7 0.077 9.4</td>
</tr>
<tr>
<td>Liver, swine</td>
<td>0.5</td>
<td>0.480 96.0 0.061 12.7</td>
<td>0.420 84.0 0.037 8.8</td>
</tr>
<tr>
<td>Liver, chicken</td>
<td>0.5</td>
<td>0.444 88.8 0.029 6.6</td>
<td>0.417 83.3 0.039 9.4</td>
</tr>
<tr>
<td>Liver, human</td>
<td>0.5</td>
<td>0.524 104.0 0.050 9.5</td>
<td>0.486 97.3 0.030 6.1</td>
</tr>
<tr>
<td>Kidney, beef</td>
<td>0.5</td>
<td>0.476 95.1 0.045 9.6</td>
<td>0.431 86.3 0.069 15.9</td>
</tr>
<tr>
<td>Heart, beef</td>
<td>0.5</td>
<td>0.459 91.8 0.049 10.7</td>
<td>0.428 85.6 0.045 10.5</td>
</tr>
<tr>
<td>Spleen, beef</td>
<td>0.5</td>
<td>0.474 94.8 0.033 7.0</td>
<td>0.414 82.7 0.027 6.6</td>
</tr>
<tr>
<td>Muscle (chuck), beef</td>
<td>0.5</td>
<td>0.443 88.6 0.077 17.4</td>
<td>0.438 87.6 0.048 11.0</td>
</tr>
<tr>
<td>Blood (coag.), beef</td>
<td>0.5</td>
<td>0.475 94.9 0.045 9.5</td>
<td>0.464 92.8 0.046 9.9</td>
</tr>
<tr>
<td>Blood (uncoag.), beef</td>
<td>0.5</td>
<td>0.418 83.6 0.054 13.0</td>
<td>0.391 78.2 0.052 13.3</td>
</tr>
</tbody>
</table>

As determined by 2-dimensional TLC and densitometry described in the text. All values represent the averages of 8–12 determinations except for human livers which represent 2 samples.

study were obtained at local supermarkets and slaughterhouses and judged aflatoxin-free after assays by the method presented in this paper. Human liver was obtained from the Pathology Department of St. Francis Hospital, Peoria, IL. Standard aflatoxin solutions (0.1 ng B\(_1\) and M\(_1\)/mL acetone) were added to blended beef liver (100 g/sample) to obtain samples spiked at levels of 0.1, 0.5, or 1.0 ng B\(_1\) and M\(_1\)/g. The same solution was added to blended or ground swine, chicken, and human liver (100 g/sample), to beef kidney, spleen, heart, muscle (chuck), and blood, and to human urine (100 g or 100 mL/sample) at a level of 0.5 ng B\(_1\) and M\(_1\)/g or mL. Both coagulated and uncoagulated (containing ethylenediaminetetraacetic acid (EDTA)) blood were tested.

**Contaminated freeze-dried liver powder.**—Contaminated dry powdered liver was prepared by thoroughly mixing standard aflatoxin solution (0.5 µg B\(_1\) and M\(_1\)/mL acetonitrile) (3.6 mL) with aflatoxin-free blended liver (3.5 kg), and the mixture was freeze-dried. The dry powder was mixed 15 min with a Hobart mixer to ensure homogeneity. Analyses were made on samples equivalent to 100 g liver by mixing 30 g powder with 70 mL water in the extraction flask.

**Extraction**

Blend meat tissue until homogeneous (muscle and heart tissues must be ground because of insufficient water content). Weigh 100 g mixture or transfer 100 mL blood or urine into 500 mL wide-mouth, glass-stopper Erlenmeyer flask (or equivalent). Add 10 mL citric acid solution and mix thoroughly with 30 cm X 1 cm glass stirring rod. After 5 min, stir again, and mix with 20 g diatomaceous earth (40 g, blood; 50 g, urine). Add 200 mL dichloromethane and stir to remove excess solids from rod. Shake flask vigorously on wrist-action shaker (setting 5 on a Burrell) for 30 min. Filter mixture through paper (588-fast flow) into 300 mL Erlenmeyer flask containing 10 g sodium sulfate. (Close filter top and compress entire filter against funnel to obtain maximum filtrate volume.). Gently swirl flask intermittently ca 2 min and refilter through paper (586 or 2V-medium flow) (588 for blood) into 250 mL graduate and record volume (cover funnel with a watch glass to prevent evaporation of solvent). Evaporate filtrate in 500 mL round-bottom flask, under vacuum, to near dryness and save for column chromatography.

**Column Chromatography**

Proceed as in 26.A13 (11) except: (1) prepare column with dichloromethane; (2) redissolve concentrated filtrate in ca 25 mL dichloromethane and add to column (use dichloromethane to rinse round-bottom flask and column); and (3) use hexane–ether–acetonitrile (6 + 3 + 1) instead of (5 + 3 + 2).

**Thin Layer Chromatography and Visual or Densitometric Analysis**

Proceed as in 26.A14c (11) for 2-dimensional TLC. Determine aflatoxin concentrations as in 26.074 and 26.031d (11), substituting (100 g or mL X filtrate vol.)/200 in formula for W.

**Results and Discussion**

Recoveries of aflatoxins B\(_1\) and M\(_1\) from artificially contaminated meat tissues and blood are given in Table 1. Generally, recoveries for B\(_1\)
Figure 1. Photograph of a 10 X 10 cm TLC plate of beef liver extract containing aflatoxins B1 and M1 (0.5 ng/g each) developed 2-dimensionally with ether-methanol-water (93 + 6 + 1) (direction 1) and chloroform-acetone-isopropanol (82 + 10 + 8) (direction 2).

were 90–95% and those for M1 were 80–85%. Coefficients of variation were 10–15% for B1 and M1 analyses. Only small differences were observed between levels (0.1–1.0 ng/g) in beef liver or between animal or tissue species. Recoveries from the 2 human livers tested were higher (104% B1 and 97% M1); however, data from only 2 samples were not representative. No unusual contaminants were observed on TLC plates for the human liver extracts. Two individual chicken livers (25–30 g each) were spiked with B1 and M1 (0.5 ng/g each), and the materials were reduced proportionately to determine if they could be assayed. Although the data are not given, individual chicken livers can be assayed successfully, but 4 livers (ca 100 g) are preferred. A comparison of the values for coagulated and uncoagulated beef blood indicated coagulated blood gave much better recoveries (95% B1 and 93% M1). This may be attributed to the swelling of red blood cells when blood is mixed with organic solvents, and either absorption, occlusion, or cleavage from bound proteins of the toxins during the extraction step. Another explanation may be that some aflatoxins form a complex with the EDTA used as an anticoagulant in the blood. The data on spiked samples indicated that coagulated blood is preferred for assay; however, this was difficult to predict without testing naturally contaminated blood samples.

The aflatoxin B1 and M1 zones on TLC plates were free of interfering substances for all samples. The cleanest extracts were obtained from blood samples. We occasionally found extraneous fluorescent zones near the B1 zone in extracts of swine liver and near the M1 zone in extracts of chicken liver; however, these impurities were not found routinely in these tissues. A typical 2-dimensional TLC plate of beef liver extract is shown in Figure 1.

A comparison of the assay results obtained with the 4 most recent analytical methods for liver (7–9) and for contaminated freeze-dried liver are presented in Table 2. The aflatoxin B1 and M1 values are very similar for all methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Aflatoxin B1, ng/g</th>
<th>Aflatoxin M1, ng/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jemmali and Murthy (7)</td>
<td>0.42</td>
<td>0.48</td>
</tr>
<tr>
<td>Truckess and Stoloff (8)</td>
<td>0.55</td>
<td>0.42</td>
</tr>
<tr>
<td>van Egmond et al. (9)</td>
<td>0.45</td>
<td>0.41</td>
</tr>
<tr>
<td>NRRC</td>
<td>0.43</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* As determined by 2-dimensional TLC and densitometry. Contaminated powdered liver prepared by mixing aflatoxins B1 and M1 with blended beef liver, and then freeze-drying. All values are an average of 2 determinations made by H. P. van Egmond, National Institute of Public Health, Bilthoven, The Netherlands.
The differences in the $M_1$ value attained by the Jemmali and Murthy method (7) and in the $B_1$ value from the Truckess and Stoloff method (8) can be attributed to fluorescent interferences in those areas of the TLC plates. Extracts from the van Egmond et al. (9) and NRRC methods had less fluorescent impurities than did the extracts from the other methods. There are large differences in the total analysis times of the 4 methods. The methods of van Egmond et al. (9) and NRRC were faster and used less solvent and glassware. Overall for analyses of meats, the NRRC method compared favorably with the other methods.

These experiments showed a noticeable increase in interferences in the freeze-dried liver extracts which were not detected with fresh liver extracts (Figure 2). The freeze-drying process caused considerable changes in the meat tissue. This made interpretation of the TLC chromatograms for these products much more difficult—especially for the aflatoxin $M_1$. Assay of frozen meat products was definitely preferred over freeze-dried.

Several observations during the development of this method should be discussed. Most of the meat tissues could be blended to achieve homogeneity, but heart and other muscle tissues have lower water contents and, therefore, should be ground with a home food grinder. Trichloroacetic acid (10%) was investigated as a denaturing agent in place of citric acid, but this resulted in neither better recoveries nor cleaner TLC extracts. Dichloromethane was used throughout the method in place of chloroform, except in the eluting solvent for column chromatography. Dichloromethane is preferred because it is less toxic than chloroform and it does not contain ethanol as a preservative. The latter was important in the dairy products collaborative study (12) because ethanol content varied (0.75–2.0%) in commercial lots of chloroform throughout the world. Concentrations greater than 0.75% led to early elution of aflatoxin from the column. Dichloromethane eliminated this problem. Chloroform in the elution solvent (acetone-chloroform ($1 + 4$)) was not changed, because no problem would exist at this stage of the cleanup; however, higher ethanol concentrations would probably elute more interferences from the column. Presumably, dichloromethane could be substituted for chloroform in the eluting solvent if 0.75% ethanol was added to ensure a similar elution volume.

A medium flow filter was important to remove fine particulate matter from the concentrated meat extracts. Failure to do so would clog the silica gel column. Preferred filter papers are S&S No. 560 or Whatman No. 2V. The column cleanup step was modified from that originally proposed for the dairy products method (10) to include a 25 mL hexane wash to remove residual.

---

Figure 2. Photograph of a $10 \times 10$ cm TLC plate of contaminated freeze-dried beef liver (0.5 ng $B_1$ and $M_1/g$) developed as described in Figure 1.
acetic acid from the column. This was necessary to prevent early elution of B₁ and M₁ from the column.

Two-dimensional TLC of the meat extracts was accomplished with 10 × 10 cm plates and required only 30 min. This saved both time and cost. It should be mentioned that on TLC plates the aflatoxin B₁ and M₁ zones from the tissue extracts have slightly lower R_f values than the standards (Figure 1). This was caused by the large concentrations of extract that were spotted on the plate. The aliquot of extract spotted represented 12-15 g of original tissue, which was considerably more than usually spotted in other aflatoxin procedures (11). Toxin migration on plates during development in the first direction was slowed by the presence of impurities in the extract. This did not prevent positive identification of the aflatoxins, but one does need to become familiar with the position of the toxins on the TLC plate. False positive samples will not be a problem because confirmatory tests (13) should be run to positively identify any suspect samples.

Aflatoxins B₂, G₁, and/or G₂ can be detected in tissue samples with this method. For quantitative data on these aflatoxins, the solvent system that resolves them best should be substituted for the second-direction solvent (isopropanol-acetone-chloroform).

This method was tried unsuccessfully with 100 mL human urine samples. Cleanup of the samples was not satisfactory, and fluorescent contaminants obscured identification of B₁ and M₁ zones at levels less than 1 ng/mL. Acid and base partitions of the urine extracts were not effective in removing the contaminants. Animal urine samples were not available to the authors, so we did not try the method on these types of samples.

The method presented in this paper was effective for analyses of most meat tissues. It was rapid, gave clean extracts for TLC, used less toxic and smaller quantities of solvents and chemicals, and was sensitive to ≤0.1 ng/g.

Acknowledgment
The authors thank J. I. Greer (NRRC) and H. P. van Egmond (Institute for Public Health, Bilthoven, The Netherlands) for analyses of samples and E. A. Emken (NRRC) for procuring human liver samples.

REFERENCES