Determination of Aflatoxins in Foods Using HPLC and a Commercial ELISA System

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ABSTRACT

A commercially available enzyme linked immunosorbent assay (ELISA) system and a high performance liquid chromatography (HPLC) method were used simultaneously to analyze 178 samples of foodstuffs for total aflatoxins. High correlation coefficients between results of the two methodologies were obtained (>0.96) with nuts, nut products, peanuts, and peanut butter. However, poor correlation was obtained from results of cereals and grain samples. The ELISA system demonstrated a high degree of reproducibility between wells (p<0.01). Of the 35 samples of nut and nut products examined, aflatoxins were detected in five samples at levels ranging from 40 to 276 μg/kg. However, of 73 peanut and peanut butter samples only one peanut sample contained 61 μg/kg, and of 70 samples of processed cereal and grain, three samples contained 6-10 μg/kg total aflatoxins. Both methods were also applied to six peanut meal samples, with known aflatoxin concentrations, provided in an interlaboratory collaborative trial. The results obtained by the HPLC and ELISA methods were in close agreement with the mean results obtained by the participating laboratories.

It is widely accepted that aflatoxins are among the most potent carcinogens and hepatotoxins occurring naturally (1). Foods such as peanuts (7,16), grains (6,9,10,14), cereals (3,16,19), and nuts (7,16) have frequently been found to be contaminated with aflatoxins.

The Pure Food Act Regulations of New South Wales (N.S.W.), Australia, set the maximum permitted level of aflatoxins at 15 μg/kg for peanuts, peanut products, nuts and the nut portion of products containing nuts, and 5 μg/kg for other foods (13). This laboratory has been engaged in the monitoring of aflatoxins (B₁, B₂, G₁, and G₂) in foodstuffs since 1981 using high performance liquid chromatography (HPLC). However, this technique has the disadvantages of being too costly, laborious, and time consuming (2,4,14).

Since its introduction in 1971 (5), the enzyme-linked immunosorbent assay (ELISA) technique has gained wide acceptance in many branches of analytical chemistry for the determination of antigens in a wide variety of substrates (2,12). This acceptance is related to the inherent simplicity, sensitivity, specificity, and high sample throughput of ELISA.

The application of immunoassay systems to the rapid detection of mycotoxins and other analytes in foods has been reviewed by Chu, Newsome, and Shepherd et al. (2,12,17). However, until the recent advent of commercial ELISA kits, the technique was only available to a limited number of research organizations.

Although several ELISA kits for aflatoxin analysis are now commercially available (11,17), there is little information regarding the relative performance of these kits against conventional HPLC techniques (2,11).

In this study, we report upon the comparative performance of a particular ELISA system, Biokits (U.K.), and a conventional HPLC method and present a summary of a survey of the incidence of aflatoxins in foods marketed in N.S.W.

Other ELISA systems were also investigated and found to be unreliable in terms of false-positive and false-negative results. These results were not reported due to space considerations.

MATERIALS AND METHODS

Samples
A total of 178 samples of peanuts, peanut butters, nuts, nut products, cereals, and grains was analyzed. These samples were obtained from retail outlets throughout N.S.W., during 1988/89 by food inspectors of the Department of Health. In addition, six artificially contaminated peanut meal samples from an interlaboratory check samples program, run by the Smalley Committee of the American Oil Chemists’ Society (AOCS), were included in this investigation.

Chemicals
Chemicals used in this investigation were of nanograde purity whenever possible; otherwise analytical reagent grade chemicals were used. Aflatoxins B₁, B₂, G₁, and G₂ were obtained from Sigma Chemical Co. (St. Louis, MO) and the ELISA kits were purchased from Biokits, Deeside, U.K. Each kit consisted of a microtiter plate with aflatoxin-coated wells, five aflatoxin B₁ standards for constructing dose-response curves, aflatoxin-free peanut meal, aflatoxin B₁ quality control concentrate, monoclonal aflatoxin antibody raised in rat, peroxidase enzyme conjugated to rabbit antirat immunoglobulin, buffers, enzyme substrate, and a solution for terminating enzyme activity. The Biokit
utilized monoclonal antibody which bound 100% of B₁ and G₁ aflatoxins as well as 60% of aflatoxins B₂ and G₂.

**HPLC determination of aflatoxins**

Total aflatoxins in the samples were determined by HPLC after extraction, partitioning, and derivatization. The method used was similar to that of Tarter et al. (18), as follows:

**Extraction.** A 50-g sample was blended in a mixture of 0.1N HCl and methanol (50 and 200 ml, respectively) for 3 min using a Waring blender. The resultant slurry was centrifuged at 700g for 10 min and the supernatant was analyzed by both the HPLC and the ELISA methods.

**Partitioning.** Of the supernatant portion, 50 ml were mixed with 50 ml of 10% NaCl then defatted with 50 ml of hexane. The aflatoxins in the aqueous layer were extracted with 2x25 ml portions of dichloromethane. The combined organic extracts were filtered through anhydrous sodium sulfate and concentrated to 1-2 ml using a Kuderna-Danish assembly on a water bath. The concentrate was then injected into a precertified Sep-Pak cartridge (Millipore) with hexane (5 ml) and rinsed with a further 2 ml of hexane followed by 5 ml of anhydrous ether. The aflatoxins retained in the cartridge were eluted into a Reacti-vial (Pierce) with 3 ml of chloroform/methanol mixture (9:1).

**Derivatization.** The solvent mixture in the Reacti-vial was evaporated under a gentle stream of nitrogen and the dried extract was mixed with 30 µl of trifluoroacetic acid. After 2 min, 970 µl of water/acetonitrile mixture (9:1) was added and the mixture was filtered through a teflon membrane filter (0.45 µm) and 50 µl of filtrate injected into the HPLC system.

**HPLC assay.** The analyses were carried out on a Hewlett Packard 1084B chromatograph connected to a Perkin-Elmer 2000 fluorescence detector (excitation at 367 nm and emission at 430 nm) and a Hewlett Packard 3392A integrator. The HPLC column used was a 250 x 4.6 mm RP-18 spheri (Brownlee). The mobile phase consisted of water/glacial acetic acid/methanol (60:20:20) and at a flow rate of 1.5 ml/min.

A mixture of the four aflatoxin standards at suitable concentrations was derivatized in the same manner as the sample. Quantitation was by an external standard method.

**ELISA determination of aflatoxins**

**Sample preparation.** A 100 µl portion of the methanolic extract used for HPLC analysis was evaporated under a gentle stream of nitrogen. The residue was dissolved in 100 µl of 50% aqueous acetonitrile then diluted with 2.4 ml of phosphate buffer.

**ELISA assay.** The assay was carried out according to the instructions provided with the kit. Briefly, 2x50 µl of each of the prepared samples, negative control, quality control, and aflatoxin standards was dispensed into the wells of the microtiter plate. Next, 50 µl of the aflatoxin antibody was added to allow the immobilized aflatoxin and the free aflatoxins to compete for binding sites on the added antibody. After 2 h of incubation at room temperature with constant mixing on an orbital shaker (Flow Laboratories), the unbound materials were removed by flicking the microtiter plate over a sink, and the wells were rinsed five times with Tris buffer. Then 100 µl of the peroxidase-immunoglobulin conjugate was added to each well, in order to determine the amount of antibody bound to the aflatoxin-coated wells. After 30 min of incubation at room temperature with constant mixing, the unbound conjugate was removed and the wells were washed five times with buffer. The substrate solution was promptly transferred to each well (100 µl) and the plate was incubated for a further 30 min with constant mixing to allow color development. The enzymatic activity was terminated by the addition of 50 µl of the reaction stopping solution which was followed by 10 s of orbital shaking. The absorbance of each well was measured at 405 nm using a microtiter plate reader (Uniskan, Flow Laboratories).

**Validation.** Validation of the ELISA kit required that it be tested for accuracy and the possibility of false responses. The accuracy of the kit was tested in two ways. Firstly, by comparing results against those obtained by our HPLC method. Secondly, by comparing results against those obtained by other laboratories using different HPLC methods in an interlaboratory collaborative trial program run by the Smalley Committee of the AOCS.

False responses were tested in two ways. Firstly, by testing the kit’s responses for samples known to contain more than the maximum recommended level of 200 ppb and up to 3000 ppb. Secondly, by testing its performance at levels near the detection limits (2 ppb). Reproducibility of the kit was evaluated by comparing response results obtained by different wells for the same sample extracts.

In order to overcome the problem associated with the heterogeneous nature of aflatoxin contamination (9,10), the HPLC and ELISA analyses were carried out on the same sample extract. Although the kit is only recommended for the analysis of edible nuts and nut products, we have also applied it to processed cereals and grains.

**RESULTS AND DISCUSSIONS**

**Survey**

The results of the analysis of 178 samples by both the conventional HPLC method and ELISA technique are presented in Table 1. Of the 73 peanut and peanut butter samples examined, only one sample of peanuts contained a total aflatoxin level that exceeded the limit permitted by the N.S.W. Pure Food Act Regulations (15 µg/kg). However, of 35 nut and nut product samples examined, five samples of all crushed mixed nuts of different origin exceeded that limit. This relatively high incidence of contamination could be attributed to poor quality control during the processing of crushed mixed nuts and suggests a need for improvements in quality control procedures. In the case of cereal and grain products, only three samples out of 70 samples examined contained total aflatoxin levels greater than 5 µg/kg, indicating a low incidence of contamination.

**Comparative performance of HPLC and ELISA**

A statistical comparison between HPLC results and ELISA results is given in Table 2. The correlation coefficient values of 0.963 for peanuts and peanut butters and 0.999 for nuts and nut products demonstrate a high degree of agreement between the two systems. In addition, the result of t-test (for paired data) showed that there was no significant difference between the results of the two systems. However, this was not the case for processed cereals and grains where the correlation coefficients obtained were 0.438 and 0.213, respectively.

The data obtained by both systems and the overall mean values obtained by about 70 participating laboratories in the AOCS interlaboratory trials are shown in Table 3. The results obtained by the HPLC method deviated from the mean obtained by the participating laboratories by less than 0.65 of a standard deviation. Three of the results obtained by the ELISA system deviated from the mean values obtained by the 70 laboratories by less than 0.5 of
TABLE 1. Total aflatoxin (B1, B2, G1, and G2) results obtained by HPLC and ELISA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of samples</th>
<th>Method of analysis</th>
<th>No. of samples containing aflatoxins in the following ranges</th>
<th>Maximum total aflatoxins found μg/kg</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>2-5</td>
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<tr>
<td>Grains</td>
<td>18</td>
<td>HPLC</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ELISA</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>Processed cereals</td>
<td>52</td>
<td>HPLC</td>
<td>34</td>
<td>16</td>
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<tr>
<td></td>
<td></td>
<td>ELISA</td>
<td>35</td>
<td>11</td>
</tr>
<tr>
<td>Nut &amp; nut products</td>
<td>35</td>
<td>HPLC</td>
<td>23</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ELISA</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>Peanuts &amp; peanut butters</td>
<td>73</td>
<td>HPLC</td>
<td>52</td>
<td>12</td>
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<tr>
<td></td>
<td></td>
<td>ELISA</td>
<td>49</td>
<td>18</td>
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</tbody>
</table>

TABLE 2. Statistical comparison of total aflatoxin results using HPLC and ELISA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of samples</th>
<th>Correlation coefficient</th>
<th>Probability P (t-test for paired data)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut and peanut butters</td>
<td>73</td>
<td>0.963</td>
<td>0.13</td>
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<tr>
<td>Nuts and nut products</td>
<td>35</td>
<td>0.999</td>
<td>0.08</td>
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<td>Processed cereals</td>
<td>52</td>
<td>0.438</td>
<td>0</td>
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<tr>
<td>Grains</td>
<td>18</td>
<td>0.213</td>
<td>0</td>
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</table>

TABLE 3. Total aflatoxin (B1, B2, G1, and G2) results for AOCS interlaboratory peanut meal samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of samples</th>
<th>DAL HPLC</th>
<th>ELISA</th>
<th>AOCS Mean ± Standard deviation</th>
<th>Deviation relative to standard deviation HPLC</th>
<th>ELISA</th>
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</thead>
<tbody>
<tr>
<td>Grains</td>
<td>18</td>
<td>213</td>
<td>176</td>
<td>185±76</td>
<td>0.37 ± 0.12</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>2</td>
<td>143</td>
<td>132</td>
<td>113±48</td>
<td>0.63</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>35</td>
<td>49</td>
<td>32±15</td>
<td>0.20</td>
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<td></td>
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<td>4</td>
<td>34</td>
<td>52</td>
<td>34±15</td>
<td>0.00</td>
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<tr>
<td></td>
<td></td>
<td>5</td>
<td>25</td>
<td>31</td>
<td>27±9</td>
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<td></td>
<td></td>
<td>6</td>
<td>48</td>
<td>60</td>
<td>43±17</td>
<td>0.29</td>
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</tbody>
</table>

*American Oil Chemist's Society.
*Results obtained by the Food Laboratory of Analytical Laboratories.
*Mean HPLC results reported by AOCS for 70 participating Laboratories.
*X - μ/standard deviation.

TABLE 4. Incidents of discrepancies between HPLC and ELISA results.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of samples</th>
<th>No. of samples HPLC-positive ELISA-negative</th>
<th>Range μg/kg</th>
<th>No. of samples HPLC-negative ELISA-positive</th>
<th>Range μg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanuts &amp; peanut butters</td>
<td>73</td>
<td>8</td>
<td>2-5</td>
<td>11</td>
<td>2-5</td>
</tr>
<tr>
<td>Nuts and nut products</td>
<td>35</td>
<td>3</td>
<td>2-4</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>Processed cereals</td>
<td>52</td>
<td>12</td>
<td>2-5</td>
<td>11</td>
<td>2-9</td>
</tr>
<tr>
<td>Grains</td>
<td>18</td>
<td>5</td>
<td>2-6</td>
<td>4</td>
<td>6-12</td>
</tr>
</tbody>
</table>

1.562 ± 0.174 in comparison to 1.559 ± 0.168 for the second well. The probability of t-test (for paired data) was 0.2426 which indicated that there was no significant difference between wells.

One of our main concerns was to determine whether or not the kit was subject to the hook effect phenomenon which is a phenomenon associated with certain immunoassay systems where a paradoxical fall in the dose-response occurs at high antigen concentration (8). The ELISA system investigated in this study produced responses indicating the presence of greater than 200 ppb for samples spiked with aflatoxins that were shown by HPLC to contain 200-3000 ppb of aflatoxins. As the operating range of this kit is 2-200 ppb, this indicated that the kit was not subject to false responses within the concentration range under investigation.

Interestingly, 28 samples that were shown by HPLC to contain aflatoxins at concentrations of 2-6 ppb failed to give a positive response with the ELISA kit (Table 4). In addition, another 28 samples shown by ELISA to contain aflatoxins at concentrations of 2-11 ppb did not give a response by HPLC (Table 4). Such discrepancies have also been reported previously (14). The concentration involved in the case of peanuts, peanut butters, nuts and nut prod-
products did not exceed 5 µg/kg. This is well below the maximum permitted level of 15 µg/kg and does not preclude the kit from being used for screening purposes of these products. However, in the case of processed cereals and grains, the discrepancies occurred at levels (2-12 ppb) which exceeded the maximum permitted level of 5 µg/kg, thus rendering the kit unsuitable for screening these products.

On the basis of the work carried out in this investigation, it is difficult to assume that those discrepancies are due to false-positive or false-negative responses by ELISA without confirmation by another technique such as GC/MS.

CONCLUSION

The results of this investigation showed that the kit can be used effectively for screening purposes of total aflatoxins in peanuts, peanut butters, nuts and nut products. The kit is most accurate in the range 15 to 50 ppb. However, in the range between 50 and 200 ppb, on a typical calibration curve, the difference in the response was limited (0.2 optical density unit, Fig. 1), and, consequently, reduced the precision and accuracy of the kit in that range. Although the kit’s manufacturers recommended that each sample should be run in duplicate, the kit demonstrated such high degree of reproducibility that we believe it is safe, for the purpose of fast screening, to test each sample using only one well. The sample throughput obtained with the kit was up to five times greater than that which could be achieved by the conventional HPLC method.

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