

# THE CONTROL OF AFLATOXIN RESIDUES IN FOOD OF ANIMAL ORIGIN

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## ABSTRACT

Apart from its presence in primary agricultural products, aflatoxin and its metabolites may be detected in animal tissues and milk. As the aflatoxins are toxic and extremely carcinogenic, control of food contamination with them is imperative. Either the primary aflatoxins ( $B_1$ ,  $B_2$ ,  $G_1$  and  $G_2$ ) or their metabolites ( $M_1$  and  $M_2$ ) have been recovered from several animal tissues and milk. The bio-assay techniques which are currently available are not suitable for routine screening purposes. The chemical assay techniques, although more accurate, faster and more specific, have many serious drawbacks particularly in relation to efficiency of extraction.

Further steps in assay procedures have been refined and standard solutions in chloroform are stable for at least 4 months at  $-10^\circ\text{C}$ . Taking into account the sensitivity of current techniques and the biological activity of aflatoxins, a level of  $1\ \mu\text{g}\ \text{kg}^{-1}$  is recommended for statutory purposes. Control in finished animal products is discussed. The most effective procedure for prevention of contamination of animal products is to exercise control of intake of the toxin.

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Aflatoxin, with its potent carcinogenic activity in many species<sup>1</sup> and widespread natural occurrence, ranks as the most important and potentially dangerous of the currently identified mycotoxins. Apart from the ingestion of aflatoxin in oilseeds, cereals and other agricultural products in which the fungus (*Aspergillus flavus*) is growing, there is the possibility of ingesting aflatoxin and its metabolic transformation products in foods on which the fungus is not growing. Foods derived from animals which are ingesting aflatoxin may contain toxic products and the potential danger to man is enhanced because the animals concerned may show no outward signs of disease.

Discussion of the methods of controlling contamination of animal products includes a consideration of the occurrence and methods of identifying the toxins.

## OCCURRENCE OF AFLATOXINS IN FOOD OF ANIMAL ORIGIN

The four most commonly found 'primary' aflatoxins in oilseeds and cereals are aflatoxin  $B_1$ ,  $B_2$ ,  $G_1$  and  $G_2$ . One or all of these compounds has been found in a wide variety of agricultural products, although groundnuts appear to be the most commonly contaminated. Because of this widespread distribution ingestion of these aflatoxins by animals is relatively common.

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Most animals metabolize the four 'primary' aflatoxins rapidly and they are found to be absent, or present in relatively low concentrations, in animal tissues or milk. Thus a lactating ewe given a dose of mixed aflatoxins ( $1 \text{ mg kg}^{-1}$ ) excreted only traces of aflatoxin  $B_1$  in the milk, although larger quantities of  $M_1$  were detected<sup>2</sup>. Similarly steers given the same dose ( $1 \text{ mg kg}^{-1}$ ) of aflatoxin  $B_1$  had traces (about  $1 \mu\text{g kg}^{-1}$ ) of aflatoxin  $B_1$  in their blood<sup>3</sup>. These are examples of the very low levels of the primary aflatoxins that have been detected.

The metabolites which have been detected in higher concentrations than the primary toxins are the aflatoxins M. These compounds have structures similar to those of the primary toxins but hydroxylated (in the liver) in the benzylic position at the junction of the two furan rings. Aflatoxin  $M_1$  and  $M_2$  are the derivatives of  $B_1$  and  $B_2$ , and  $GM_1$  and  $GM_2$  are the derivatives of  $G_1$  and  $G_2$  respectively<sup>4,5</sup>. As aflatoxin  $B_1$  is normally the aflatoxin occurring in high concentration in agricultural products, aflatoxin  $M_1$  is the commonly found metabolite.

Aflatoxin M was originally found in cow's milk<sup>6</sup> and has subsequently been found in ewe's<sup>2</sup> and goat's<sup>5</sup> milk. The amount present in milk is proportional to the intake<sup>3,5,7-9</sup> and levels of up to  $50 \mu\text{g l}^{-1}$  have been reported. Aflatoxin M has not been detected in bulk milk supplies<sup>6,10</sup>, but was found in retail milk from primary groundnut-producing areas in South Africa<sup>11</sup>.

Aflatoxin M occurs at lower levels in body tissues, with the majority of reports indicating that it is undetectable in meat, blood, fat, etc. It has been reported in the tissues of steers<sup>3</sup> and chickens<sup>12</sup> given large doses of aflatoxin. Recent results indicate that in certain circumstances pigs receiving a diet containing relatively small amounts of aflatoxin can have significant residues of toxin in body tissues<sup>13</sup>.

There are other metabolites of aflatoxin which have been described in laboratory animals. Thus the demethoxylated derivative (aflatoxin P) has been recovered from monkey urine<sup>14</sup> and a metabolite which appears to be non-toxic has been recovered from avian liver homogenates<sup>15</sup>. The latter metabolite was subsequently shown to be 'aflatoxicol'<sup>16</sup>. Neither of these metabolites has been reported to occur in human food.

## SIGNIFICANCE OF AFLATOXIN RESIDUES

Residues of aflatoxin  $B_1$  in food must be considered dangerous in view of the toxicity and extreme carcinogenicity of this compound. Similarly aflatoxins  $B_2$ ,  $G_1$  and  $G_2$ , although less toxic and carcinogenic than aflatoxin  $B_1$ , are dangerous and should not be present in foods.

Aflatoxins  $M_1$  and  $M_2$  have the same acute toxicity to day-old ducklings as the parent compounds aflatoxin  $B_1$  and  $B_2$ <sup>17</sup>, and aflatoxin  $M_1$  is carcinogenic<sup>11,18</sup>. The same care should thus be taken to ensure that aflatoxin  $M_1$  does not occur in foods.

As far as the other metabolites are concerned, there is little evidence on their toxicity. Aflatoxicol is relatively non-toxic<sup>19</sup> but no studies of its carcinogenicity have been reported. The presence of aflatoxicol or other non-toxic metabolites in food should be viewed with concern, as this indicates that other, possibly more toxic, metabolites may be present.

## IDENTIFICATION OF AFLATOXIN RESIDUES

## Biological assay

The day-old duckling has been used as a test animal for determining the presence of aflatoxin<sup>20</sup>, with the degree of proliferation of bile ducts as a quantitative index. Wogan<sup>21</sup> has shown that it is at best semiquantitative and that a dose of 0.4 µg aflatoxin B<sub>1</sub> per duckling per day for 5 days (total dose 2.0 µg) is required to produce bile duct changes. This test is likely to be effective for detecting aflatoxin M<sub>1</sub> as the bile duct changes are quantitatively similar to those produced by aflatoxin B<sub>1</sub><sup>17</sup>. Little information is available on the sensitivity of this test to other metabolites of aflatoxin. Aflatoxicol is much less toxic to ducklings<sup>19</sup> as are other derivatives, such as aflatoxin B<sub>2a</sub><sup>22</sup>, and it can be expected that there will be a wide variation in the response of ducklings to compounds produced by modifications in the aflatoxin molecule.

Table 1. Summary of methods described for assaying aflatoxin in animal tissues. Estimation of quantity of toxin in each method follows on t.l.c. separation and is based on fluorescence

| Reference                                       | Substrate        | Extracting solvents   | Clean-up                                 | Sensitivity             | Comments                                  |
|---|------------------|---|--|-------------------------|---|
| Allcroft and Carnaghan (1963) <sup>6</sup>      | Milk and tissues | MeOH (Soxhlet)  | Solvent partition                        | ?                       | Semi-quantitative                         |
| de Jongh <i>et al.</i> (1964) <sup>35</sup>     | Milk powder      | MeOH 2h<br>CHCl <sub>3</sub> 3h (Soxhlet)                       | Solvent partition                        | ?                       | Semi-quantitative                         |
| Van der Linde <i>et al.</i> (1964) <sup>7</sup> | Liquid milk      | MeOH  | Solvent partition                        | ?                       | Semi-quantitative                         |
| Heusinkveld <i>et al.</i> (1965) <sup>30</sup>  | Meat             | Acetone-hexane-H <sub>2</sub> O column azeotrope (blend)        | Florosil                                 | ?                       | Originally for peanuts; semi-quantitative |
| Purchase and Steyn (1967) <sup>27</sup>         | Milk powder      | Acetone-CHCl <sub>3</sub> -H <sub>2</sub> O azeotrope (Soxhlet) | Pb acetate then solvent partition        | ?                       |   |
| Allcroft and Roberts (1968) <sup>8</sup>        | Liquid milk      | Boil with acetone   | Solvent partition                        | ?                       | Semi-quantitative                         |
| Masri <i>et al.</i> (1968) <sup>9</sup>         | Milk powder      | MeOH-H <sub>2</sub> O (blend)                                   | Solvent partition then silica gel column | ?                       |   |
| Bullerman <i>et al.</i> (1969) <sup>31</sup>    | Meat             | CHCl <sub>3</sub> (blend)                                       | Silica gel column                        | ?                       | For B <sub>1</sub> and G <sub>1</sub>     |
| Van Zytveld <i>et al.</i> (1970) <sup>12</sup>  | Meat             | CHCl <sub>3</sub> (blend)                                       | Silica gel column                        | ?                       | Semi-quantitative                         |
| Jacobsen <i>et al.</i> (1971) <sup>26</sup>     | Liquid milk      | MeOH (blend)  | Solvent partition then celite column     | 0.1 µg kg <sup>-1</sup> | B <sub>1</sub> + M <sub>1</sub>           |
| Asplin and Carnaghan (1961) <sup>20</sup>       | Milk and others  | Biological testing  | -day-old ducklings                       | 2 µg per duckling       | Semi-quantitative                         |
| Meyer <i>et al.</i> (1969) <sup>23</sup>        | Milk             | Biological testing—maize seedlings                              |  | 0.1 µg in 0.01 ml       | Semi-quantitative                         |

The prevention of virescence in 12-day-old maize seedlings has been used as a biological test for the presence of aflatoxin B<sub>1</sub> and M<sub>1</sub> in milk<sup>23</sup>. The sensitivity of this method in milk powder is about 0.15 µg aflatoxin B<sub>1</sub> equivalents per gramme.

These two biological tests are not used extensively, particularly for screening purposes, because they are expensive, time consuming, relatively non-specific and only semiquantitative.

### Chemical assay methods

A summary of published methods for aflatoxins is given in *Table 1*. All methods use the comparison of intensity of fluorescence of a standard with that of the sample for estimation of the quantity of toxin present.

### Standard solutions

Problems have been encountered with standards of aflatoxin M<sub>1</sub>, particularly with respect to keeping quality.

A recent study<sup>24</sup> has shown that the use of ethanol or methanol accelerates the deterioration of aflatoxin M standards particularly when stored in soft (soda) glass or nylon tubes. Better storage conditions are provided by solutions in chloroform, or benzene-acetonitrile (98:2) in pyrex (borosilicate) glass containers. These findings have been confirmed and extended by the recent IUPAC collaborative study<sup>25</sup> on the stability of aflatoxin M<sub>1</sub> standards. In this study aflatoxin M<sub>1</sub> standards were prepared in chloroform and in benzene-acetonitrile. After 3 months storage at -10°C there was a negligible change in the concentration in the chloroform-based standards although assays on the benzene-acetonitrile-based standards were variable due to insolubility of the aflatoxin M<sub>1</sub>. From these studies the recommendation for keeping aflatoxin M<sub>1</sub> standards is to use chloroform as the solvent in borosilicate glass containers at low temperatures (-10°C). Under these conditions aflatoxin M<sub>1</sub> standards are stable for at least 4 months.

### Problems of assessing the suitability of assay techniques

The first step in an assay technique, namely the extraction of the toxin, is most difficult to assess accurately. The two separate facets which should be investigated are the efficiency and reproducibility of the extraction technique. The easiest method of assessing both parameters is to 'spike' samples with a known amount of the toxin, as Jacobsen *et al.*<sup>26</sup> have done. In this way the accuracy and precision may be determined. There is, however, the drawback that aflatoxin added to an animal product in an organic solvent will be in a different physical form from that present by virtue of ingestion. Some scanty evidence to support this contention is available. Treatment of milk powder with chloroform does not extract all the aflatoxin M present and the residue cannot be extracted with solvents which are normally effective<sup>27</sup>. This may be related to the fact that there is a higher concentration of aflatoxin in milk than in venous blood and, therefore, that the mechanism for transporting aflatoxin to the milk requires a special physical form (e.g. bonded to amino acids). Whatever the reason is, some doubt remains about whether the accuracy and precision of a method determined on spiked samples represents

efficiency and reproducibility in the extraction of 'naturally' occurring toxin.

In one study the efficiency of various extracting solvents for Soxhlet extraction and extraction by blending with milk powder were compared<sup>27</sup>. The wide variation in efficiency of these solvents suggests that the solvents used in many of the methods, although providing reproducible results, may be inaccurate because of under-estimation. A further example is that methanol was found to extract larger quantities of aflatoxin B from tissues than the acetone-chloroform-water azeotrope but the results were much more variable<sup>28</sup>.

An alternative method of assessing the efficiency of extraction methods is to use duckling bio-assay to determine whether any residual aflatoxin is present in the extracted sample. This technique has been used to assess residues after extraction by two techniques and in both cases some biological activity was found<sup>29</sup>. The drawback here is that the nature of the biologically active substances is unknown, and there may have been other metabolites of aflatoxin which were unextractable but biologically active.

Many investigators have used assay methods which were originally described for assaying aflatoxin B<sub>1</sub> in agricultural products (e.g. Refs. 12, 30, 31). The drawback is that the extracting solvents, which are effective for aflatoxin B<sub>1</sub> in agricultural products, may not be effective for aflatoxin M in animal products. Methanol, which extracts more aflatoxin B from tissues than does the acetone-chloroform-water azeotrope, is less effective than the azeotrope in extracting aflatoxin M from the same tissue<sup>28</sup>.

Further steps in the assay methods, including clean-up and quantification of the toxin, can be adequately studied on spiked samples, and effective methods have been described.

It may be deduced from the above discussion that assay methods for these toxins in animal products have not been studied in sufficient detail to determine their optimum accuracy and precision. Future studies should include (a) a comparison of the extracting solvent with solvents used in other published methods, (b) an assessment of the method on 'naturally' contaminated products as well as 'spiked' samples, (c) a careful evaluation of the method for both the primary toxins and their metabolites, (d) a separate evaluation of the clean-up and assay procedure, and (e) an evaluation of the sensitivity of the method.

## CONTROL MEASURES

As a first step in controlling the presence of aflatoxin in animal products rapid and accurate assay techniques are required to identify and quantify the toxins. None of the published methods can be said to fulfill all the requirements of a method for the statutory control of aflatoxins in animal products. More work will be required to refine the existing methods. The sensitivity of these methods also has to be considered. There are no safe levels for a potent carcinogen such as aflatoxin but the limitation of assay techniques must be taken into account. Current techniques are able to detect levels of  $1 \mu\text{g kg}^{-1}$  in a relatively small sample and this is below the lowest concentration in food known to produce tumours in the most sensitive experimental animals<sup>1</sup> and lower than the minimum concentration which will affect tissue

cultures<sup>32</sup>. It is, however, greater than the minimum no-effect dose of aflatoxin B<sub>1</sub> in the feed of rainbow trout<sup>33</sup>. This level (0.05 µg kg<sup>-1</sup>) is several times lower than similar doses in other species. Thus a level of 1 µg kg<sup>-1</sup> would seem to be a reasonable limit at the present stage, but this should be reviewed when more sensitive methods are available.

## PREVENTION OF CONTAMINATION

### Destruction

Although Allcroft and Carnaghan<sup>6</sup> reported that heat treatment of milk did not alter the toxicity of extracts of milk powder, a subsequent study using a more refined chemical assay technique showed that the quantity of toxin decreased considerably on heat treatment. The aflatoxin M content of milk (containing 385 µg kg<sup>-1</sup> powder) was reduced to 140 µg kg<sup>-1</sup> on pasteurization at 80°C, to 72 µg kg<sup>-1</sup> on sterilization and to 52 µg kg<sup>-1</sup> on spray drying<sup>34</sup>. These alterations were also detectable using duckling bio-assay<sup>34</sup> and may thus be considered to be due to conversion of aflatoxin M into non-toxic products.

Heat treatment of milk (by pasteurization) or meat (by cooking) is thus likely to produce a marked reduction in the aflatoxin M content. In situations where no other control procedures can be applied, heat treatment is likely to provide some degree of control, although it is certainly not ideal.

### Dilution

Surveys of bulk milk supplies have failed to detect aflatoxin M contamination<sup>6,10</sup>, probably due to dilution of any contamination. Although this cannot be considered a desirable control method, it is one which occurs by virtue of modern processing techniques.

### Prevention

The concentration of aflatoxin B or M in animal products is less than 0.1 per cent of the ingested concentration. As there is a limit to the level which can be ingested without deleterious effect to the animal, the amount present in products of the animal are likely to be low in most practical situations. It follows that statutory and practical control of contamination will be much easier to apply to feedstuffs than the final animal products. Where detectable levels of aflatoxin are allowed in animal feeds care should be taken to ensure that the animals are placed on aflatoxin-free rations some time before slaughter. Most aflatoxin appears to be excreted in 24 to 48 hours, but for safety's sake a longer period should be recommended.

In the case of animals producing milk for human consumption, stricter control is necessary. As there is a linear relationship between intake of aflatoxin B<sub>1</sub> and excretion of aflatoxin M<sub>1</sub>, one would expect that the intake of even small amounts of aflatoxin B<sub>1</sub> would result in the presence of aflatoxin M<sub>1</sub> in milk even if the level is below that which can be assayed by current methods. Therefore, the level in feedstuffs for lactating animals should be reduced to a 'negligible' level, i.e. a level below that which can be

detected by current assay methods. In situations where ingestion of aflatoxin has occurred, the milk should be discarded for 2–5 days.

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