# EXTRACTION AND DETERMINATION OF *ASPERGILLUS FLAVUS* METABOLITES—AFLATOXINS FROM MEAT PRODUCTS†

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## ABSTRACT

On the basis of the many methods for the extraction and determination of aflatoxins from cereals a method of aflatoxin extraction in meat products was elaborated. Methanol–water and chloroform solvents were used for the extraction, and a petroleum ether–benzene–hexane mixture was used for defatting the samples. Thirty different combinations of solvents were selected to find the best of them for developing t.l.c. plates. Separation of the interfering substances of meat extracts from aflatoxins and resolution of four aflatoxins ($B_1$, $B_2$, $G_1$ and $G_2$) were obtained when a combination of hexane, petroleum ether, benzene, chloroform and acetone was used.

## INTRODUCTION

Some *A. flavus* strains are toxinogenic. They can grow on several meat products especially on durable fermented sausages and smoked meats. Salami and smoked cured hams are foods that fall into this category.

Moulds from fermented sausages obtained in the USA, Hungary, Italy, Spain, Holland, Germany, Switzerland, Greece, Romania, Yugoslavia and Czechoslovakia were isolated by Ayres *et al.*[1], and Leistner and Ayres[13].

Fungi from 'country cured ham' obtained in the states of Missouri, Kentucky, Iowa, Virginia, Tennessee, North Carolina, and Georgia were isolated by Ayres *et al.*[1] and Strzelecki *et al.*[16].

In both types of products, moulds of the genera *Rhizopus*, *Mucor*, *Mortierella*, *Syncephalastrum*, *Penicillium*, *Aspergillus*, *Scopulariopsis*, *Paecylomyces*, *Oospora*, *Cladosporium*, *Alternaria*, *Epicoccum* and *Fusarium* were found. Members of the Aspergillus group consisted of *A. flavus*, *A. ochraceus*, *A. tamarii*, *A. ruber*, *A. repens*, *A. amstelodami*, *A. chevalieri*, *A. pseudoglaucus*, *A. maginin* and *A. peniciloides*. Of the 10 fungi isolated by Strzelecki *et al.*[16], from a heavily moulded country cured ham, 4 were identified as toxinogenic strains of *Aspergillus flavus*.

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In the past many methods have been used for the extraction and determination of aflatoxins from products of plant origin. There is a strong possibility that aflatoxins can be found in food products of animal origin just as they are found in cereals, especially when processing and storage practices result in a suitable environment for mould growth. The elaboration of a method for the extraction and determination of aflatoxins in meat products therefore seemed purposeful.

## MATERIAL AND METHODS

Salami, raw ham and country cured ham were used as the materials for this work. Extractions of meat extract, or meat extract combined with standard aflatoxins which were added to the samples before extracting them, were made on 100 g meat pieces.

For defatting the samples and extraction of aflatoxins several solvents or combinations of them were used. *Table 1* shows the method for the extraction of aflatoxins from meat, which was elaborated on the basis of the above studies.

The first 3 speeds of a 4-speed Osterizer were used to blend the sample for 1–2 min at each speed. The sample consisted of 100 g of meat in 300 ml of methanol in distilled water (2:1). The sample was filtered through a 12.7 cm (5 in) Büchner funnel with Whatman No. 1 filter paper and 50 g of anhydrous sodium sulphate. After filtering, 200 ml of a petroleum ether–hexane–benzene solvent (2:1:2) and 10 g of sodium chloride were added to the filtrate and shaken in a separating funnel. The methanol–water layer was transferred to another separating funnel and the petroleum ether–hexane–benzene layer discarded. This process was repeated 3 times. The combined methanol–water layer was extracted four times with 25 ml aliquots of chloroform. Each aliquot was collected separately in a 100 ml beaker and passed through a 5 cm (2 in) aluminium oxide column to which 5 cm (2 in) of anhydrous sodium sulphate was added. Neutral Brockmann No. 1 aluminium oxide was packed dry in a 15 × 300 mm column. Each aliquot was allowed to pass through the column to a 5 mm level before the next was added. The beaker was washed and the column eluted with 100 ml of 5 per cent methanol in chloroform which was collected in a 500 ml round-bottom flask. The eluate was then evaporated to dryness on a Buchler rotary evaporator. The residue was cooled under running water and dissolved in 5 ml of chloroform. The detection and quantification of aflatoxins from this extract were performed by t.l.c. using the procedure outlined by Pons *et al.*[14]. For the separation of the aflatoxins from the other fluorescent materials and for the resolution of four aflatoxins ($B_1$, $B_2$, $G_1$ and $G_2$) different solvents were investigated. The combinations of the developing solvents were prepared from acetic acid, acetone, benzene, carbon tetrachloride, chloroform, ethanol, ethyl ether, hexane, methanol and petroleum ether in the proportions shown in *Table 2*.

## RESULTS AND DISCUSSION

Many methods have been elaborated for the investigation of aflatoxins in cereals. The first preparative step in those studies involved cutting, grinding

*Table 1.* Scheme of the method for the extraction and detection of aflatoxins from meat products

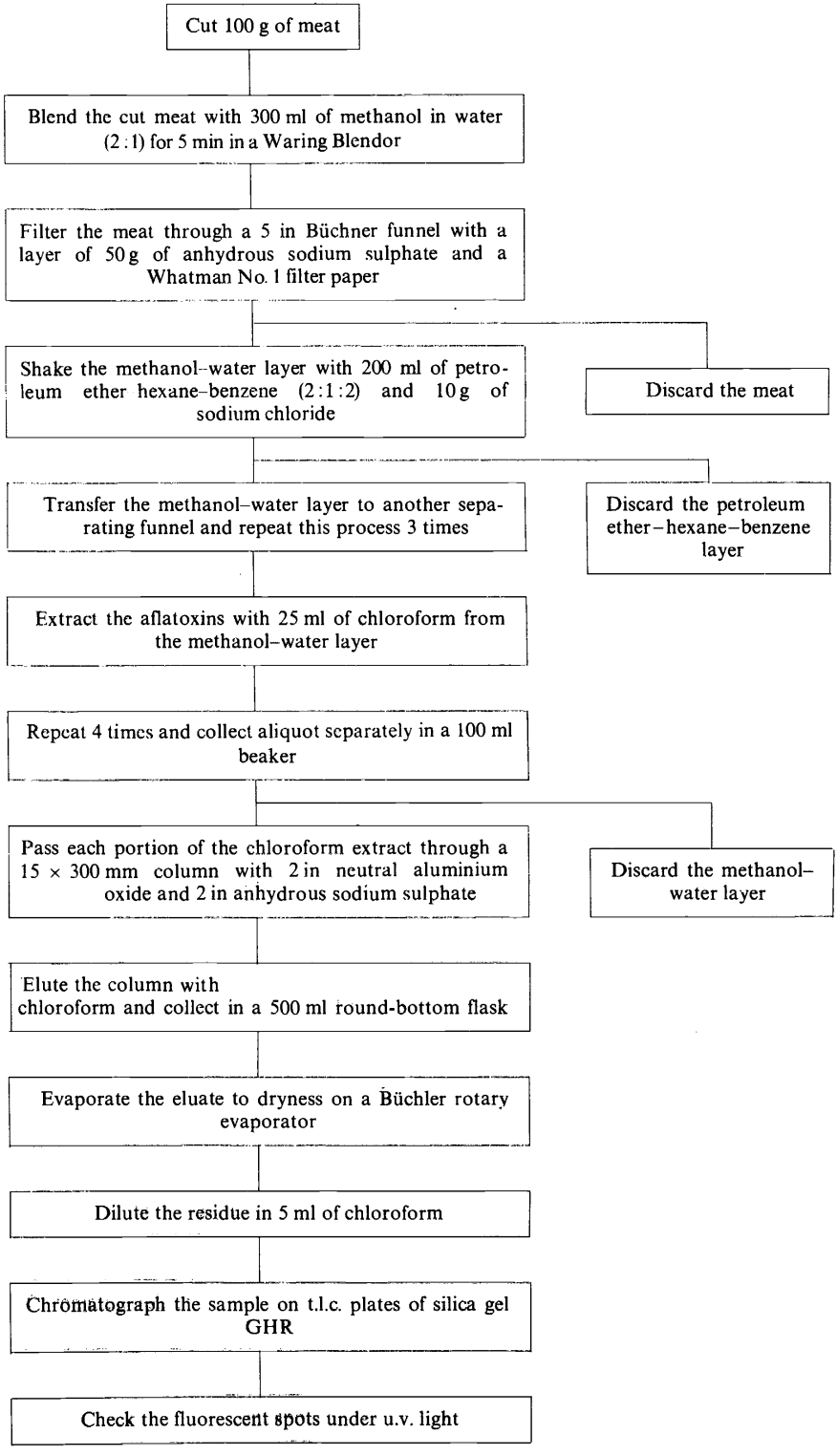

*Table 2.* Developing solvents for thin-layer chromatography of standard aflatoxins combined with meat extracts

| No. | Solvent | Proportion | Viscosity, 20°C (cP) | Solubility parameter (partition eluent strength) | Relative absorbent eluent strength* | Separation of aflatoxins from fluorescent substances | Resolution of aflatoxins B–G | Resolution of aflatoxins $B_1-B_2-G_1-G_2$ |
|---|---|---|---|---|---|---|---|---|
| 1 | Carbon tetrachloride | 50 | 0.97 | 8.6 | IW | | | |
| | Acetic acid | 50 | 1.22 | 10.1 | S | – | – | – |
| 2 | Carbon tetrachloride | 50 | 0.97 | 8.6 | IW | | | |
| | Acetone | 50 | 0.32 | 9.9 | IS | – | – | – |
| 3 | Carbon tetrachloride | 50 | 0.97 | 8.6 | IW | | | |
| | Ethanol | 50 | 1.19 | 12.7 | S | – | – | – |
| 4 | Carbon tetrachloride | 50 | 0.97 | 8.6 | IW | | | |
| | Methanol | 50 | 0.59 | 14.4 | S | – | – | – |
| 5 | Chloroform | 90 | 0.56 | 9.3 | I | | | |
| | Acetone | 10 | 0.32 | 9.9 | IS | + | + | + |
| 6 | Chloroform | 97 | 0.56 | 9.3 | I | | | |
| | Methanol | 3 | 0.59 | 14.4 | S | – | – | – |
| 7 | Chloroform | 91 | 0.56 | 9.3 | I | | | |
| | Methanol | 9 | 0.59 | 14.4 | S | – | – | – |
| 8 | Hexane | 50 | 0.32 | 7.3 | W | | | |
| | Acetic acid | 50 | 1.22 | 10.1 | S | – | – | – |
| 9 | Hexane | 50 | 0.32 | 7.3 | W | | | |
| | Acetone | 50 | 0.32 | 9.9 | IS | – | – | – |
| 10 | Hexane | 50 | 0.32 | 7.3 | W | | | |
| | Ethanol | 50 | 1.19 | 12.7 | S | – | – | – |
| 11 | Hexane | 50 | 0.32 | 7.3 | W | | | |
| | Methanol | 50 | 0.59 | 14.4 | S | – | – | – |
| 12 | Hexane | 10 | 0.32 | 7.3 | W | | | |
| | Chloroform | 80 | 0.56 | 9.3 | I | – | – | – |
| | Methanol | 10 | 0.59 | 14.4 | S | | | |
| 13 | Hexane | 40 | 0.32 | 7.3 | W | | | |
| | Ethyl ether | 30 | 0.23 | 7.4 | I | + | – | – |
| | Acetone | 30 | 0·32 | 9.9 | IS | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 14 | Hexane | 20 | 0.32 | 7.3 | W | | | |
| | Petroleum ether | 20 | — | — | — | + | + | + |
| | Chloroform | 50 | 0.56 | 9.3 | I | | | |
| | Acetone | 10 | 0.32 | 9.9 | IS | | | |
| 15 | Hexane | 30 | 0.32 | 7.3 | W | | | |
| | Carbon tetrachloride | 15 | 0.97 | 8.6 | IW | | | |
| | Ethyl ether | 10 | 0.23 | 7.4 | I | − | − | − |
| | Acetone | 15 | 0.32 | 9.9 | IS | | | |
| | Methanol | 30 | 0.59 | 14.4 | S | | | |
| 16 | Hexane | 40 | 0.32 | 7.3 | W | | | |
| | Ethyl ether | 40 | 0.23 | 7.4 | I | | | |
| | Chloroform | 5 | 0.56 | 9.3 | I | − | + | + |
| | Acetone | 10 | 0.32 | 9.9 | IS | | | |
| | Methanol | 5 | 0.59 | 14.4 | S | | | |
| 17 | Hexane | 65 | 0.32 | 7.3 | W | | | |
| | Ethyl ether | 15 | 0.23 | 7.4 | I | | | |
| | Chloroform | 7 | 0.56 | 9.3 | I | + | + | − |
| | Acetone | 10 | 0.32 | 9.9 | IS | | | |
| | Methanol | 3 | 0.59 | 14.4 | S | | | |
| 18 | Hexane | 30 | 0.32 | 7.3 | W | | | |
| | Ethyl ether | 30 | 0.23 | 7.4 | I | | | |
| | Chloroform | 5 | 0.56 | 9.3 | I | + | − | − |
| | Acetone | 30 | 0.32 | 9.9 | IS | | | |
| | Methanol | 5 | 0.59 | 14.4 | S | | | |
| 19 | Hexane | 30 | 0.32 | 7.3 | W | | | |
| | Ethyl ether | 45 | 0.23 | 7.4 | I | | | |
| | Chloroform | 10 | 0.56 | 9.3 | I | + | + | − |
| | Acetone | 10 | 0.32 | 9.9 | IS | | | |
| | Methanol | 5 | 0.59 | 14.4 | S | | | |
| 20 | Hexane | 10 | 0.32 | 7.3 | W | | | |
| | Petroleum ether | 10 | — | — | — | | | |
| | Benzene | 10 | 0.65 | 9.2 | I | + | + | + |
| | Chloroform | 60 | 0.56 | 9.3 | I | | | |
| | Acetone | 10 | 0.32 | 9.9 | IS | | | |

* W, weak; IW, intermediately weak; I, intermediate; IS, intermediately strong; S, strong

| No. | Solvent | Proportion | Viscosity, 20°C (cP) | Solubility parameter (partition eluent strength) | Relative absorbent eluent strength* | Separation of aflatoxin from fluorescent substances | Resolution of aflatoxins B–G | $B_1-B_2-G_1-G_2$ |
|---|---|---|---|---|---|---|---|---|
| 21 | Hexane | 30 | 0.32 | 7.3 | W | – | + | – |
| | Benzene | 25 | 0.65 | 9.2 | I | | | |
| | Ethyl ether | 15 | 0.23 | 7.4 | I | – | + | – |
| | Chloroform | 10 | 0.56 | 9.3 | I | | | |
| | Acetone | 10 | 0.32 | 9.9 | IS | | | |
| | Methanol | 10 | 0.59 | 14.4 | S | | | |
| 22 | Hexane | 30 | 0.32 | 7.3 | W | | | |
| | Benzene | 30 | 0.65 | 9.2 | I | | | |
| | Ethyl ether | 30 | 0.23 | 7.4 | I | + | + | – |
| | Chloroform | 3 | 0.56 | 9.3 | I | | | |
| | Acetone | 4 | 0.32 | 9.9 | IS | | | |
| | Methanol | 3 | 0.59 | 14.4 | S | | | |
| 23 | Hexane | 20 | 0.32 | 7.3 | W | | | |
| | Benzene | 20 | 0.65 | 9.2 | I | | | |
| | Ethyl ether | 20 | 0.23 | 7.4 | I | – | + | – |
| | Chloroform | 10 | 0.56 | 9.3 | I | | | |
| | Acetone | 20 | 0.32 | 9.9 | IS | | | |
| | Methanol | 10 | 0.59 | 14.4 | S | | | |
| 24 | Hexane | 30 | 0.32 | 7.3 | W | | | |
| | Benzene | 20 | 0.65 | 9.2 | I | | | |
| | Ethyl ether | 30 | 0.23 | 7.4 | I | + | + | – |
| | Chloroform | 5 | 0.56 | 9.3 | I | | | |
| | Acetone | 10 | 0.32 | 9.9 | IS | | | |
| | Methanol | 5 | 0.59 | 14.4 | S | | | |
| 25 | Hexane | 20 | 0.32 | 7.3 | W | | | |
| | Carbon tetrachloride | 20 | 0.97 | 8.6 | IW | | | |
| | Ethyl ether | 20 | 0.23 | 7.4 | I | – | + | – |
| | Chloroform | 20 | 0.56 | 9.3 | I | | | |
| | Acetone | 10 | 0.32 | 9.9 | IS | | | |
| | Methanol | 10 | 0.59 | 14.4 | S | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 26 | Hexane | 30 | 0.32 | 7.3 | W | | | |
| | Carbon tetrachloride | 10 | 0.97 | 8.6 | IW | | | |
| | Ethyl ether | 35 | 0.23 | 7.4 | I | | | |
| | Chloroform | 10 | 0.56 | 9.3 | I | + | + | − |
| | Acetone | 10 | 0.32 | 9.9 | IS | | | |
| | Methanol | 5 | 0.59 | 14.4 | S | | | |
| 27 | Hexane | 30 | 0.32 | 7.3 | W | | | |
| | Carbon tetrachloride | 15 | 0.97 | 8.6 | IW | | | |
| | Ethyl ether | 35 | 0.23 | 7.4 | I | | | |
| | Chloroform | 10 | 0.56 | 9.3 | I | − | + | + |
| | Acetone | 5 | 0.32 | 9.9 | IS | | | |
| | Methanol | 5 | 0.59 | 14.4 | S | | | |
| 28 | Hexane | 20 | 0.32 | 7.3 | W | | | |
| | Carbon tetrachloride | 20 | 0.97 | 8.6 | IW | | | |
| | Ethyl ether | 20 | 0.23 | 7.4 | I | | | |
| | Chloroform | 15 | 0.56 | 9.3 | I | − | − | − |
| | Acetone | 20 | 0.32 | 9.9 | IS | | | |
| | Methanol | 5 | 0.59 | 14.4 | S | | | |
| 29 | Hexane | 20 | 0.32 | 7.3 | W | | | |
| | Ethyl ether | 20 | 0.23 | 7.4 | I | | | |
| | Chloroform | 20 | 0.56 | 9.3 | I | | | |
| | Acetone | 20 | 0.32 | 9.9 | IS | − | − | − |
| | Ethanol | 10 | 1.19 | 12.7 | S | | | |
| | Methanol | 10 | 0.59 | 14.4 | S | | | |
| 30 | Hexane | 60 | 0.32 | 7.3 | W | | | |
| | Ethyl ether | 20 | 0.23 | 7.4 | I | | | |
| | Chloroform | 4 | 0.56 | 9.3 | I | | | |
| | Acetone | 10 | 0.32 | 9.9 | IS | + | + | − |
| | Methanol | 3 | 0.59 | 14.4 | S | | | |
| | Acetic acid | 3 | 1.22 | 10.1 | S | | | |

* W, weak; IW, intermediately weak; I, intermediate; IS, intermediately strong; S, strong

or blending the sample (Campbell and Funkhouser[2], Engebrecht *et al.*[6], and Eppley[7,8]), followed by defatting with hexane, diethyl ether, petroleum ether or benzene (Eppley[9] and Trager *et al.*[17]). Aflatoxins were extracted with methanol, methanol–water, chloroform, chloroform–water, acetone–water, acetone–hexane, acetone–hexane and water, or acetone–methanol and water (Chen and Friedman[3], Holaday[11], Lee[12], Pons *et al.*[14] and Robertson *et al.*[15]). These extractions were carried out in Soxhlet apparatuses, centrifuges, shakers, or separating funnels (Campbell and Funkhouser[2], Coomes and Sanders[4], and Eppley[7,8]). Eppley[7] separated solids from liquids by passing the mixture through filter paper covered with a layer of anhydrous sodium sulphate. A saturated sodium chloride solution was added by Robertson *et al.*[15] to eliminate emulsions. The extracted aflatoxins were then purified by column chromatography. Adsorbents used were celite 545, cellulose powder CF 1, silica gel and Alumina Woehlm Neutral (Chen and Friedman[3], Pons *et al.*[14] and Trager *et al.*[17]). Chloroform–methanol or chloroform–hexane mixtures were used by Campbell and Funkhouser[2], and Trager *et al.*[17] in eluting the aflatoxins from the columns.

In this study the samples of meat were cut and blended, and then defatted by a solvent mixture of petroleum ether, hexane and benzene. Other solvents did not improve the defatting of the meat samples. The extraction of the aflatoxins was performed by the use of a methanol–water solvent during the blending of the sample and then chloroform extraction from the methanol–water layer in a separating funnel to which sodium chloride was added before shaking. Purification of the aflatoxins was achieved by use of column chromatography. Aluminium oxide covered with anhydrous sodium sulphate was used as the column packing. This procedure was chosen as it appeared to be the most rapid.

A recovery of 0.003–0.0006 μg to 0.0003–0.0004 μg aflatoxin is claimed by Coomes *et al.*[5] and Trager *et al.*[17] using t.l.c. Total recovery of the aflatoxins varied from 0 to 100 per cent in different laboratories (Campbell and Funkhouser[2], and Chen and Friedman[3]).

In this work the recovery of the aflatoxins was performed on meat samples taken from raw ham, country cured ham and salami. Aflatoxins were added to the meat samples before blending; different levels were used. The amounts of aflatoxin $B_1$ were 0.2, 1.0, 5.0, 25.0 and 125.0 μg per 100 g of meat for each type of meat sample, but aflatoxins $B_2$, $G_1$ and $G_2$ were added in different amounts depending upon the standard that was used. For the raw ham the average recovery was 66.6 per cent for aflatoxin $B_1$ and 64.4 per cent for the total amount of aflatoxins. Aflatoxin $B_1$ was found in 70.3 per cent and 62.2 per cent in country cured ham and salami respectively, but the total amount of aflatoxins recovered was 67.9 per cent and 59.1 per cent respectively.

Trager *et al.*[17] separated aflatoxins by thin-layer chromatography on silica gel GHR, Alumina Woelhm Neutral, or Alumina G Merck. Chloroform–methanol, chloroform–acetone, and benzene–ethanol and water were frequently used as solvents for developing chromatograms by Eppley[7], Trager *et al.*[17], and others.

In this study the prepared meat extracts were spotted on t.l.c. plates coated with a 0.25 mm thickness of silica gel GHR. On each t.l.c. plate, 10 μl of standard aflatoxin, 10 μl of meat extract and 10 μl of meat extract combined

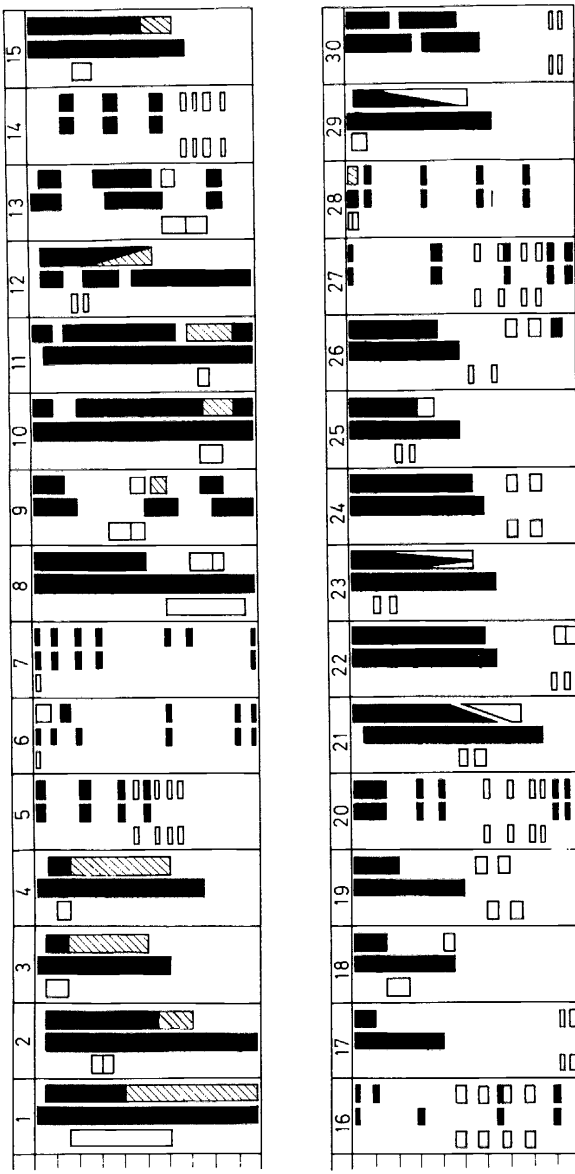


*Figure 1.* Schematic presentation of interfering substances of meat extracts with aflatoxin standards on t.l.c. plates. (See *Table 2* for correspondingly numbered solvent combinations)

with 10 µl of internal standard aflatoxins were spotted. This was repeated three times with each sample of meat extract. The t.l.c. plates were then developed in 30 different combinations of developing solvents as presented in *Table 2* and *Figure 1*.

The concentrations of the various spots were determined visually by comparing with aflatoxin standards obtained from the Southern Utilization Research and Development Laboratory, USDA, New Orleans, La. The separation of standard aflatoxin from meat extract was achieved in 11 cases, the resolution of two aflatoxins (B and G) was obtained in 15 cases, and the resolution of four aflatoxins ($B_1$, $B_2$, $G_1$ and $G_2$) was obtained in 5 cases. However, the separation of interfering substances and the resolution of aflatoxins were not obtained in the same combinations of solvents in all cases. Only 3 combinations of solvents gave the proper separation and resolution. The first combination which gave the separation and resolution was chloroform–acetone (used by some authors). In this combination of solvents a fluorescent compound of meat extract was set between aflatoxin $B_1$ and $B_2$. For this reason a combination of the solvents hexane, petroleum ether, chloroform and acetone was much better because all fluorescent materials appeared below or above the standard aflatoxins. However, in spite of the fact that a fluorescent substance of meat extract was above the standard aflatoxin, it was too close to aflatoxin $B_1$. Since it was supposed that the fluorescent substance was a fat compound, benzene was added to the above solvent combination. This enabled the separation of fluorescent materials from the meat extract and the resolution of four aflatoxins to be achieved. It seems that for the above separation and resolution a combination of weak, intermediate or intermediately strong solvents (Heftmann[10]) should be used. Also the solubility parameter should not be higher than 10.0.

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## REFERENCES

[1] J. C. Ayres, D. A. Lillard and L. Leistner, Proceedings 20th Annual Reciprocal Meat Conference, Am. Meat Sci. Assoc., Lincoln, Nebr., June 14–16 (1967).
[2] A. D. Campbell and J. T. Funkhouser, *J. Assoc. Offic. Anal. Chemists*, **49**, 730 (1966).
[3] S. C. Chen and L. Friedman, *J. Assoc. Offic. Anal. Chemists*, **49**, 28 (1966).
[4] T. J. Coomes and J. C. Sanders, *Analyst*, **88**, 209 (1963).
[5] T. J. Coomes, P. C. Crowther, B. J. Francis and L. Stevens, *Analyst*, **90**, 492 (1965).
[6] R. H. Engebrecht, J. L. Ayres and R. O. Sinhuber, *J. Assoc. Offic. Anal. Chemists*, **48**, 815 (1965).
[7] R. M. Eppley, *J. Assoc. Offic. Anal. Chemists*, **49**,473 (1966).
[8] R. M. Eppley, *J. Assoc. Offic. Anal. Chemists*, **49**, 1218 (1966).
[9] R. M. Eppley, *J. Assoc. Offic. Anal. Chemists*, **51**,74 (1968).

[10] E. Heftmann, *Chromatography*, Reinhold, New York (1967).
[11] C. E. Holaday, AACC–AOCS Joint Meeting, March 31–April 4, Washington, D.C. (1968).
[12] W. V. Lee, *Analyst*, **90**, 305 (1965).
[13] L. Leistner and J. C. Ayres, *Fleischwirtschaft*, **48**, 62 (1968).
[14] W. A. Pons, Jr., A. F. Cucullu, L. S. Lee, J. A. Robertson, A. O. Franz and L. A. Goldblatt, *J. Assoc. Offic. Anal. Chemists*, **49**, 554 (1966).
[15] J. A. Robertson, Jr., L. S. Lee, A. F. Cucullu and L. A. Goldblatt, *J. Am. Oil Chemists' Soc.*, **42**, 467 (1965).
[16] E. L. Strzelecki, H. S. Lillard and J. C. Ayres, *Appl. Microbiol.*, **18**, 938 (1969).
[17] W. T. Trager, L. Stoloff and A. D. Campbell, *J. Assoc. Offic. Anal. Chemists*, **47**, 993 (1964).