Journal of Chromatography, 354 (1986) 305–315
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 284

COMPARISON AND CRITICAL EVALUATION OF SIX PUBLISHED EXTRACTION AND CLEAN-UP PROCEDURES FOR AFLATOXIN M_1 IN LIQUID MILK

MARTIN J. SHEPHERD, MICHAEL HOLMES and JOHN GILBERT*
Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Haldin House, Queen Street, Norwich NR2 4SX (U.K.)
(Received October 14th, 1985)

SUMMARY

A practical evaluation has been carried out of six previously published extraction and clean-up methods for aflatoxin M_1 in liquid milk. The procedures evaluated incorporated the most widely used stages of clean-up including solvent extraction and silica gel chromatographic clean-up, selective solvent extraction of the extracted residue, the use of deproteination prior to hydrophilic column liquid–liquid partition or solvent extraction and the use of pre-packed reversed-phase cartridges for the direct extraction of aflatoxin M_1 from the milk. Analysis times for each method, recoveries and relative costs are reported together with fluorescence high-performance liquid chromatography chromatograms, obtained under identical conditions to compare the relative cleanliness of the final extracts produced by each method. A pre-packed reversed phase cartridge method was shown to be the most satisfactory in terms of speed, cost and cleanliness of the final residue.

INTRODUCTION

There continues to be considerable interest in aflatoxin M_1 contamination of dairy products as evidenced by the number of papers reporting survey results[1–3] and further stimulated by the imposition of various national statutory limits[4,5]. There have been a number of recent papers[6–14] proposing new analytical methods for aflatoxin M_1 and the analyst involved in routine monitoring needs continually to reassess his choice of a particular procedure in the light of possible improved techniques. For arbitration of any possible dispute over aflatoxin M_1 levels in a particular commodity, or for critical samples one is necessarily obliged to employ methods like that of the AOAC[15] which have been thoroughly collaboratively tested. However for routine surveillance and for quality control the most rapid methods available need to be employed, provided adequate specificity can be achieved through efficacious extraction and clean-up stages. In this laboratory over the course of several years, we have routinely employed a modified Stubblefield extraction and clean-up method[16], for the analysis of several hundred samples of milk for aflatoxin M_1, using

0021-9673/86/$03.50

both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) for the final determination[1]. This method has proved to be reliable and reasonably rapid and has given good results when employed in the U.K., the European Economic Community (Community Bureau of Reference), and internationally organised collaborative trials.

Recent publications[9–14] do however suggest that alternative procedures might offer the advantages of more rapid analyses, reduced consumption of expensive solvents, and cleaner final extracts. A comparison of these methods and assessment of the various claims is not always easy without carrying out experimental work as the final determination step may be different in each case and criteria like ease and speed of analysis can to some extent be subjective. Therefore, in order to evaluate alternative methodologies for aflatoxin M_1 in liquid milk we have undertaken a critical practical appraisal of six published procedures. The results of this assessment are reported in this paper.

Although there have been a large number of methods published for aflatoxin M_1, the differences between procedures are often only slight and the six methods evaluated in this paper were chosen as being representative of a number of different principles which have been employed in the extraction and clean-up stages. These methods are summarised in Table I. The Stubblefield method[16] is based on chloroform extraction of milk and silica gel column clean-up, whilst the Chang and DeVries method[9] employs a similar extraction of the milk but selective solvent extraction of the residue replaces the chromatographic stage. The Gauch *et al.* method[10] was chosen to exemplify the use of hydrophilic liquid–liquid partition columns (Extrelut), whilst Chambon *et al.*[11] have employed a deproteination step prior to solvent extraction. The Ferguson-Foos and Warren procedure[12] uses a pre-packed Sep-Pak C_{18} cartridge for the extraction from the milk followed by a silica gel column clean-up, whilst the Takeda method[13] is similar in its use of a reversed-phase Sep-Pak for the extraction but the choice of different conditions avoids the need for any further clean-up.

TABLE I

SUMMARY OF AFLATOXIN M_1 METHODS UNDER EVALUATION

Method	*Volume of milk (ml)*	*Preliminary treatment*
Stubblefield[16]	50	None
Chang and DeVries[9]	50	None
Gauch *et al.*[10]	50	Hydrochloric acid (0.4 ml), methanol (15 ml)
Chambon *et al.*[11]	20	Zinc sulphate–sodium hydroxide deproteination
Ferguson-Foos and Warren[12]	20	Warm water (20 ml)
Takeda[13]	10	Water (10 ml)

These methods have been variously developed for both TLC and HPLC determinations, but as HPLC is generally less demanding of the cleanliness of the final extract because of its increased resolution over TLC, this has been employed under identical conditions for the analysis of all the final extracts. Thus after gaining experience with each method, and modifying (but only where absolutely necessary) to facilitate operation in our hands, the speed of analysis, unit cost, recovery, and cleanliness of the final extract have been assessed under identical conditions and are reported in this paper together with a commentary on the methods.

EXPERIMENTAL

Materials

Pasteurized bulked commercial whole milk which contained less than 0.005 μg/kg of the toxin was stored frozen and a thawed aliquot spiked with a solution of aflatoxin M_1 in acetonitrile to give the desired concentrations. The same supply of milk was used throughout to avoid any differences in potential interferences, although spiking in each case was carried out immediately before analysis. Aflatoxin M_1 standard was obtained from Sigma (Poole, U.K.), and quantitative solutions were standardised using the recommended procedure[15]. Sep-Pak C_{18} and Extrelut pre-packed columns were obtained from Waters Chromatography Division of Millipore (London, U.K.) and BDH (Poole, U.K.), respectively.

Methods

Full detailed descriptions of each method are given in the original publications, but for comparative purposes the principle of each is outlined here together with an indication of any modifications that were found necessary to operate the methods successfully in our hands. Any such modifications were kept to the absolute minimum.

Stubblefield method[16]. This involves chloroform extraction of milk by shaking

Extraction	*Clean-up*	*Published recovery (%)*	*Published determination*
Chloroform (120 ml)	Silica-gel column	80	TLC (one dimension)
Chloroform (120 ml)	Acetonitrile–light petroleum extract	100 ± 13	HPLC (TFA derivative)
Extrelut column	Eluate (dichloromethane–toluene) washed with sodium hydroxide and hydrochloric acid	78 ± 4	TLC (two developments)
Chloroform extract of filtrate	None	83–89	HPLC
Sep-Pak C_{18}	Silica gel column	99–103	HPLC (silica gel packed flow cell)
Sep-Pak C_{18}	None	72–95	HPLC (TFA derivative)

together with saturated sodium chloride solution, evaporating the solvent to dryness and clean-up on a silica gel column with three solvent washings prior to elution with acetone–chloroform. Modifications were as described elsewhere[1], involving a reduction in the volume of sodium chloride solution added to the milk to 5 ml while the chloroform extractant was not heated to 35°C. The hexane wash was increased to 15 ml and elution of M_1 was with acetone–chloroform (2:3) rather than (1:4).

Chang and DeVries method[9]. This entails extraction of aflatoxin M_1 into chloroform by gentle rolling of the milk with the solvent, evaporation of the chloroform to dryness and then extraction of the residue into acetonitrile, washing with light petroleum and evaporation to dryness, redissolving in chloroform prior to analysis. The only modifications that were introduced were extraction for 10 min rather than the 3 min proposed in the method and the replacement of light petroleum with hexane.

Gauch et al. method[10]. This stipulates milk protein precipitation with concentrated hydrochloric acid, heating to assist coagulation and then extraction of the filtrate on an Extrelut column. The eluate in dichloromethane–toluene is washed with sodium hydroxide and hydrochloric acid prior to analysis. No modifications were introduced.

Chambon et al. method[11]. This requires deproteination of the milk by the addition of zinc sulphate and sodium hydroxide followed by filtration. The filtrate is washed with hexane (this step has been mistakenly left out of the written procedure given in ref. 11 although it may be inferred from the text) and then extracted into chloroform, evaporating to dryness. The method was followed exactly but it was found necessary to record the volume of chloroform recovered (usually 60–80%) and to use this to correct the final recovery.

Ferguson-Foos and Warren method[12]. This is based on extraction of toxin by passage of the warmed milk, diluted with water, through a reversed-phase Sep-Pak previously primed with methanol, washing and then eluting with diethyl ether. This is followed by a clean-up on a silica gel column using vacuum elution of the aflatoxin in methylene chloride–ethanol solvent.

Slight modifications were made in that only 10 ml of milk were extracted (as opposed to the recommended 20 ml) as this was found simpler to pass through the Sep-Pak, and also loading onto the cartridge was under syringe pressure rather than the proposed vacuum system. A number of the silica gel columns were extracted simultaneously using a vacuum manifold system which speeded up this stage of the clean-up.

Takeda method[13]. This relies upon extraction of the aflatoxin M_1 by passing the diluted milk through a reversed-phase Sep-Pak C_{18}, washing with firstly 10% basic acetonitrile, then 10% acidic acetonitrile and finally eluting with 30% acidic acetonitrile. No changes were introduced into this method.

HPLC analysis

All results were obtained under optimum conditions for aflatoxin separations[17], using a Varian 5500 ternary chromatograph and a 250 × 4.9 mm I.D. 5-μm Spherisorb ODS column, maintained at 35°C and eluted with water–acetonitrile–methanol (60:30:10) at a flow-rate of 0.75 ml/min. The solvents were purged with helium and a back-pressure regulator adjusted to 2 bar was attached to

the outlet of the fluorimeter. A Waters in-line filter with a 2-μm frit was placed between the injector (Rheodyne 7125 with a 20-μl loop) and the column. Fluorescence detection was employed using a Perkin Elmer Model LS-4 spectrofluorimeter with the excitation and emission monochromators set at 355 and 433 nm respectively, as indicated by stopped-flow scanning of an M_1 standard.

Batches of six samples were analysed in all cases, consisting of duplicates each of a blank, a 0.016-μg/kg spike and a 0.16-μg/kg spike. All sample residues were made up immediately prior to analysis in a volume of water–acetonitrile (80:20) such that 100 μl represented 25 ml of milk. Aliquots (10 μl) of these solutions were injected onto the HPLC system using the partial loop-fill method in order to conserve sample. Results were displayed on a chart recorder at a speed of 5 mm/min under identical conditions for each method of analysis. Calculations were made on a peak height basis.

RESULTS AND DISCUSSION

One of the main difficulties of evaluating methods is that familiarity with a given procedure tends to be prejudicial, and for this reason each new method was carried out many times during the course of several weeks, and repeated until we were satisfied that it was performing reproducibly and with good recovery according to the claims of the originators of the method. Assessment to some extent has been relative to the Stubblefield method, as this procedure had been employed in these laboratories for several years for aflatoxin M_1 analyses and has given clean extracts for both TLC and HPLC determinations. The only problems with the Stubblefield method has been that in inexperienced hands the initial extraction can give rise to emulsions, and in addition we have not found it to be a particularly rapid procedure, a batch of six milk samples usually taking a complete day for analysis including the determination step and allowing time for preparation of columns and reagents.

In Table II the analysis time per sample is given for the six methods together with recoveries, detection limits and the relative costs of the different procedures. The two most rapid procedures were those based on reversed-phase Sep-Pak extraction of aflatoxin from the milk (Ferguson-Foos and Warren, and Takeda methods) and these took less than half the time of the Stubblefield method to carry out. Generally the more rapid methods were those which were also less expensive on solvent and other material requirements, although in assessing overall relative costs of different methods the labour element is often far more important. With high assigned labour costs, the order of total costs was identical to the order in which methods are placed in terms of analysis times.

The recoveries (Table II) were generally acceptable, ranging from 60 to 84%, based on milk spiked at 0.16 μg/kg. Similar values were found at the lower concentration, 0.016 μg/kg, for all methods except for that of Gauch *et al.*, where the recovery fell to 42%. All results quoted are averages from four samples (the duplicate milks spiked at 0.16 μg/kg in both of the last two batches analysed using each method). The higher detection limit of the Gauch *et al.* method was a direct consequence of its lower recovery. This was attributed to losses during the alkaline wash stage in the procedure which although carried out rapidly as stipulated in the method might still give rise to degradative losses of aflatoxin M_1. It should however be noted

TABLE II

CRITICAL COMPARISON OF AFLATOXIN M_1 METHODS

Method	*Analysis time per sample (h)*	*Average recovery (%)*	*Limit of detection (μg/kg)*	*Relative costs (solvents/materials)**	*Total relative costs (including labour)**
Stubblefield[16]	4	77	0.005	1.3	2.2
Chang and DeVries[9]	2.5–3.0	80	0.005	1.7	1.7
Gauch *et al.*[10]	5.5–6.0	60	0.01	4.0	3.5
Chambon *et al.*[11]	3.5–4.0	74	0.005	1.0	2.1
Ferguson-Foos and Warren[12]	1.5	84	0.005	1.8	1.0
Takeda[13]	1.5–2.0	70	0.005	1.2	1.2

* Costs are calculated relative to the lowest cost per method. The cost of labour is estimated at 3.7 times greater per hour than unit cost of solvent/materials.

that this method is reported to give satisfactory results with a wide range of dairy products, including powdered milks, baby food and cheeses. Low recoveries of chloroform were encountered during the extraction stage of the Chambon *et al.* method and recovered volumes were recorded in order to correct for losses at this stage.

Manipulative difficulties were experienced with the large volumes of solvent applied to the Extrelut columns in the Gauch *et al.* method and the columns themselves seemed to be prone to leaking around the tips, requiring constant attention by the operator, although this might be attributable to our lack of experience with this technique. A general disadvantage in methods like that of Takeda and that of Gauch

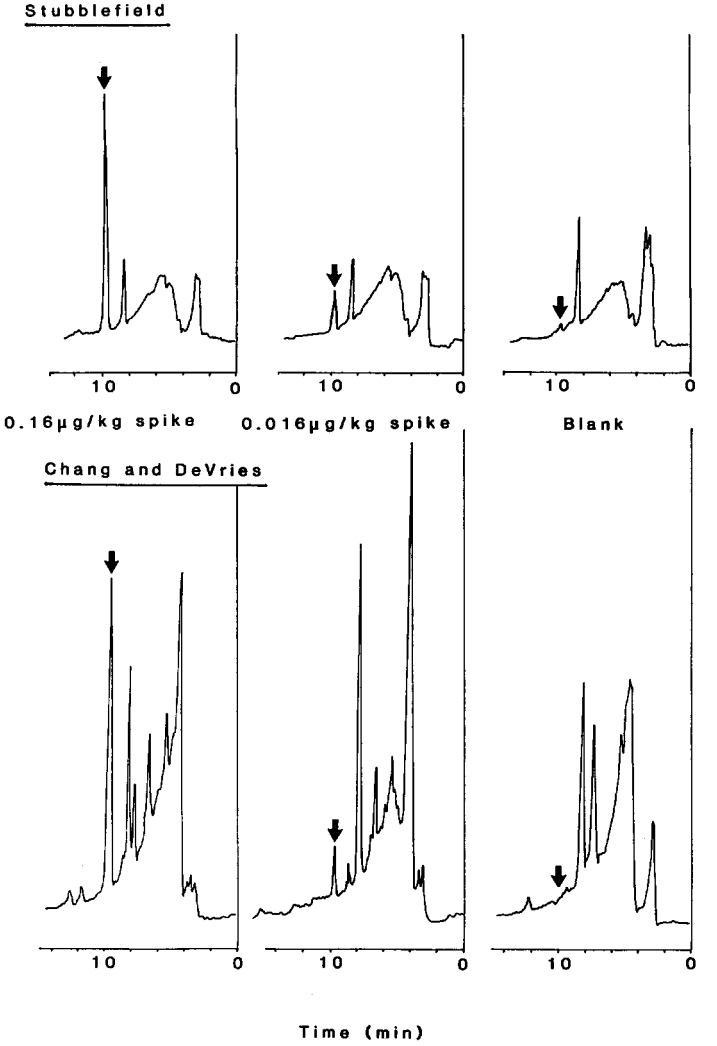

Fig. 1. HPLC fluorescence chromatograms illustrating the clean-up for aflatoxin M_1 (arrows) by the Stubblefield[16] and the Chang and DeVries[9] methods. HPLC conditions as described in the Experimental section.

et al. was that final solvents were water or other high boiling point solvents such as toluene, when the final evaporation to dryness may be the most time-consuming step in the procedure (depending upon the efficiency of the vacuum pump used) and also potentially cause losses of aflatoxin M_1. This difficulty is avoided in the method of Ferguson-Foos and Warren where the additional chromatographic stage allows transfer of the extract into a more suitable final solvent for evaporation. An additional partition stage could be added to the Takeda method to transfer the toxin into chloroform[18] but it is doubtful whether this would be cost effective. Four of the methods eliminate direct solvent extraction of the milk, a procedure which can be a source of emulsion difficulties and low recoveries, but the deproteination stages in-

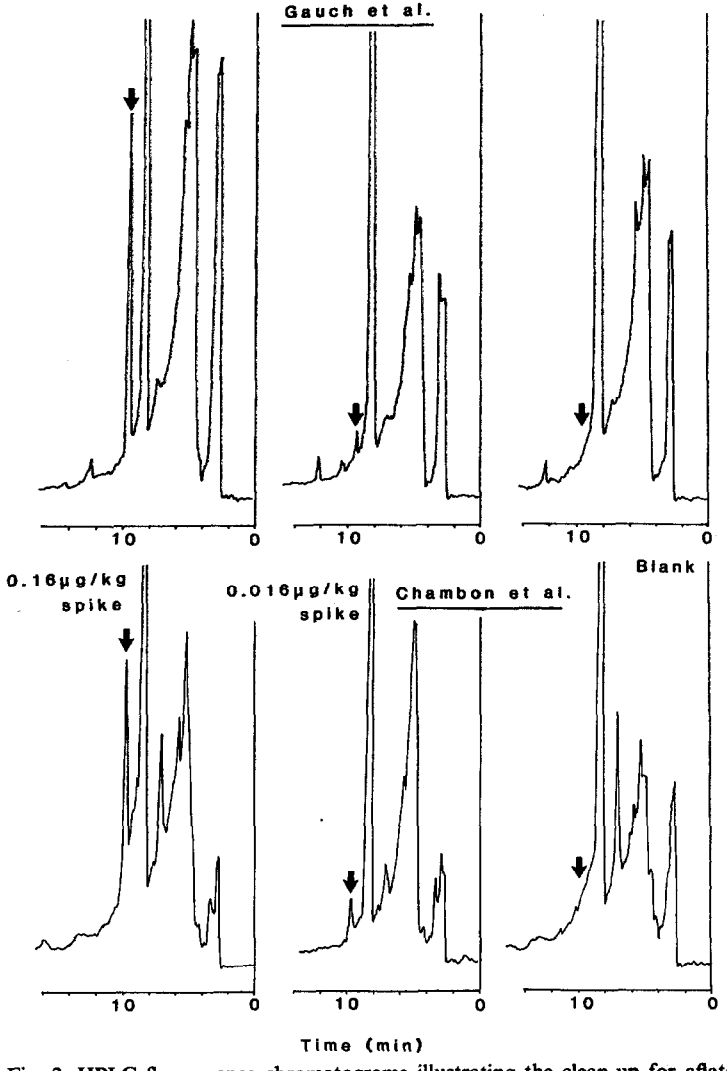

Fig. 2. HPLC fluorescence chromatograms illustrating the clean-up for aflatoxin M_1 (arrows) by the Gauch *et al.*[10] and Chambon *et al.*[11] methods. HPLC conditions as described in the Experimental section.

volved in the Gauch *et al.* and the Chambon *et al.* methods are themselves critical and therefore seem to offer little advantage. In contrast using a prepacked cartridge for the extraction stage results in a much simpler operation, allowing significant time savings over the solvent extraction, and this is a considerably attractive aspect of both the Takeda and the Ferguson-Foos and Warren procedures.

One major constraint upon sensitivity is the quality of fluorescence detector employed. For the LS-4 with a new lamp, 2 pg of M_1 injected on column could be detected at a signal-to-noise ratio of 3:1. However, the critical feature in the assess-

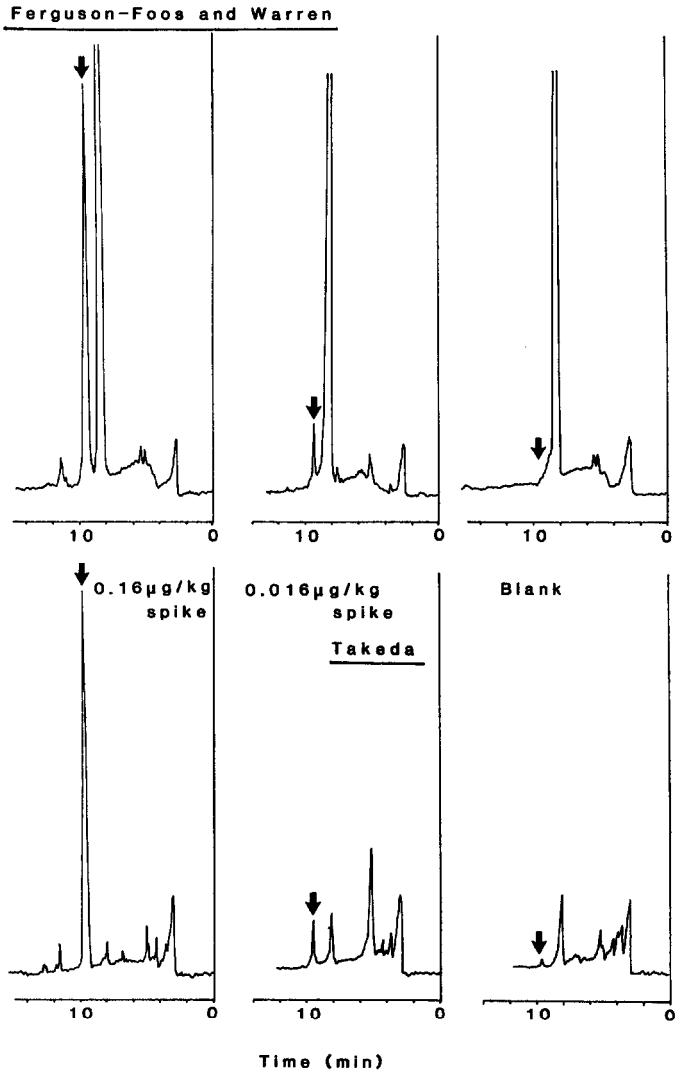

Fig. 3. HPLC fluorescence chromatograms illustrating the clean-up for aflatoxin M_1 (arrows) by the Ferguson-Foos and Warren[12] and the Takeda[13] methods. HPLC conditions as described in the Experimental section.

ment of any method of analysis for aflatoxin M_1 is the cleanliness of the final extract, in terms of background noise components affecting the limit of detection and the likely extent of interferences giving rise to false positives. The detection limits of the six methods (Table II) were broadly similar although, as discussed above, the Gauch *et al.* method gave a slightly higher value due to the lower recovery obtained. The HPLC chromatograms for milk blanks, and for spikes into milk at 0.016 μg/kg and 0.16 μg/kg are shown for each of the six methods in Figs. 1–3. The Chang and DeVries, Gauch *et al.*, and Chambon *et al.* methods in our view give unacceptable background components in the extracts which although not directly interfering with the aflatoxin M_1, do mean that the peak is eluting on the tail of a comparatively large extraneous component. The Ferguson-Foos and Warren, and the Takeda methods both give chromatograms that are cleaner than the Stubblefield method in the initial region of the trace, but the Ferguson-Foos and Warren method does give a rather large additional component eluting immediately before aflatoxin M_1, which might be a source of some difficulty if operating under less than optimum HPLC conditions. Overall the Takeda method gave the cleanest chromatogram, with the blank showing possibly 0.002 μg/l of M_1, a level also observable in the Stubblefield trace, although not confirmed. It is of interest that only the later two methods produced chromatograms where such a low level of contamination would be observable.

We did not employ trifluoroacetic acid (TFA) derivative formation prior to HPLC (unlike Takeda) because the fluorescence detector employed here gives high sensitivity without the factor of three enhancement found for the TFA reaction product. A further consideration is that the derivative elutes much earlier than M_1 from the HPLC column, in an area of the chromatogram where potential interferences are often found. Confirmation of positive results is however best achieved by formation and chromatography of the aflatoxin M_1–TFA product, and it is therefore encouraging that the Takeda method gives good results for both the toxin and its derivatives.

CONCLUSIONS

The evaluation exercise discussed in this paper has clearly demonstrated the benefits of a Sep-Pak procedure for the extraction and clean-up of aflatoxin M_1 from milk, both in terms of time savings and also improved cleanliness of the final extract. In our view the Takeda method was clearly shown to be the preferable method, bearing in mind the constraints of our assessment procedure, primarily that only one particular milk has been examined. In addition, this method should be more amenable to automation than other procedures. It should be noted that the AOAC is currently collaboratively testing the Ferguson-Foos and Warren method[19]. We intend in the future to adopt the Takeda method for the routine analysis of aflatoxin M_1 in milk and milk related products; preliminary results indicate that this method is also suitable for the analysis of milk powders. However, the ultimate test of any method is continued use over a period of time, and only after routine application can our initial assessment finally be confirmed.

ACKNOWLEDGEMENT

We are grateful to U. Leuenberger, R. Gauch, P. Chambon and N. Takeda for their comments on the manuscript.

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