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MODERN METHODS FOR AFLATOXIN M, DETECTION IN MILK AND DAIRY PRODUCTS

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Modern Methods for Aflatoxin M_1 Detection in Milk and Dairy Products

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INTRODUCTION

In the science of mycotoxins, three main disciplines play a role of major importance: mycology, toxicology and chemistry. Although the title of this lecture identifies (analytical) chemistry as the discipline to which attention will be focused, it is appropriate to start this introduction with some background on the contamination of dairy products with mycotoxins, in casu aflatoxin M_1, and on the reasons why so much attention on this subject is being paid.

Mycotoxins may contaminate dairy products by moulds growing on them or by the carry-over of mycotoxins occurring in animal feedstuffs ingested by dairy cattle. An example of the first mentioned possibility is the formation of sterigmatocystin, a carcinogenic mycotoxin, on hard cheese (Northolt, 1980). The second possibility, the carry-over, is considered to be of major importance, as it was found that aflatoxin B_1, the most notorious of the mycotoxins, is converted by the dairy cow into its 4-hydroxy derivative, aflatoxin M_1 (fig.1)

which appears in the milk (Allcroft, 1963). Experiments with cows have shown that 1-4% of the ingested aflatoxin B_1 can be recovered in the milk as aflatoxin M_1. From experimental work with rainbouw trout, there are indications that aflatoxin M_1 is carcinogenic, although probably less potent than aflatoxin B_1 (Canton, 1975; Sinnhuber, 1974), Hsieh (1982) conducted a chronic study with two groups of fifty male Fisher rats which were continuously fed diets containing 50 g M_1/kg and 50 g B_1/kg, respectively, for 18 months. At the end of this period, multihepatocarcinomas were found in 100% of the B_1-group, whereas no evidence of neoplasm was found in any of the M_1-group. Despite this, the question as to whether Maflatoxin M_1 must be considered as a carcinogen is difficult to answer, as more extensive studies than those mentioned have not been possible, because of the lack of sufficient pure material. On the basis of its structural resemblance to aflatoxin B_1, toxicologists consider it possible that aflatoxin M_1 has carcinogenic properties. Therefore, the presence of this compound is considered to be undesirable in food. In consequence, some countries have enacted legal measures to control M_1 contamination of milk and milk products. From an international inquiry (Schaller, 1983) it became clear that, until 1981, only Belgium, the Netherlands, Switzerland and the United States has established tolerance levels for M_1 in milk at 1, 0.05, 0.01-0.05 and 0.5 g/kg respectively. However, in various other countries the feasibility of establishing tolerances for aflatoxin M_1 contamination of milk has been discussed.

Because of the undesirability of aflatoxin M_1 in food much scientific attention has been focused to the occurrence of aflatoxin M_1 in dairy profucts, to the effects of processing on aflatoxin M_1, to the development and improvement of methods of analysis to determine aflatoxin M_1 and last but not least to possibilities to eliminate or prevent aflatoxin M_1 contamination. Before going to the main theme of this lecture, the analytical methofology, we shall first have a closer look to the occurrence of aflatoxin M_1 in dairy products and to the effects of processing. The prevention of M_1 contamination of milk and milk profucts is mainly a matter of prevention of aflatoxin B_1 formation or decontamination of B_1 contaminated commodities. As the latter subject will be discussed in a separate lecture by Dr.Ciegler, no further attention to it will be given in this presentation.

OCCURRENCE

In many countries of the western hemisphere milk is monitored routinely for aflatoxin M_1. As an example, the results of a survey of 105 samples of milk, collected in the Netherlands in the winter period of 1981 (Van Egmond, 1982a) are presented in figure 2, as a histogram, with class intervals of 0.03 g/litre. In 84 of 105 samples, aflatoxin M_1 could be detected in levels ranging from 0.015 to 0.09 g/litre. The mean M_1 content in raw, as well as in heat-treated, milk was 0.03 g/litre, the median M_1 content for both types of milk was also 0.03 g/litre.

The results of a similar survey, carried out in 1972, are presented in figure 3. In 1972 ca. 18% of the samples had an M_1 content of 0.1 g/litre, the Dutch action level; in 1981 none of the samples had an M_1 content exceeding this level. This significant decrease in the M_1 contamination is probably the effect of the EEC Directive (Anon., 1974) on permitted levels for undesirable substances and products in feedingstuffs. In this directive, which came into force in 1976, a year in-between the two surveys, a maximum concentration of 20 g aflatoxin B_1/kg supplementary feedingstuff for dairy cows was tolerated. Contrary to this effect in a serious recent increase of M_1-levels in the Dutch milks in the last winterperiod, due to relatively high levels of B_1 in the feedingstuffs. The feedingstuff regulations in the EEC have been further tightened to 10 g aflatoxin/kg since 1 January 1984, and it is hoped that the M_1-levels will decrease again.

Data of surveys carried out in other European countries, e.g. in France (Fremy, 1982), indicate M_1-levels, comparable to those determined in Dutch milks. In countries where feedingstuff regulations are more stringent, e.g. in Switzerland, lower M_1 levels occur in the milk.

EFFECTS OF PROCESSING ON AFLATOXIN M_1

The frequent occurrence of aflatoxin M_1 in milk has raised the question: what happens if such contaminated milk is processed in the normal manner by the dairy industry? There have been several investigations into this subject, one

of which was carried out in our Institute in close co-operation with the Netherlands Institute for Dairy Research (Van Egmond, 1977). In that study, milk, naturally contaminated with M_1, was either pasteurized or sterilized, and yogurt and cheese were prepared. The results of the study are presented in figure 4. In that figure the length of the bars represents the M_1 content found. The aflatoxin M_1 content of milk is not reduced by heat treatments like pasteurization and sterilization, nor was any reduction noticed in the M_1 content during the preparation of yogurt and cheese. It is of interest to note that the M_1 concentration in cheese was about four times higher than in the milk from which it was prepared. Accordingly, the M_1 concentration in the whey was proportionally lower. Furthermore, the various yogurt-cultures and cheese starters did not influence the M_1 content.

More recent studies were carried out by Brackett (1982) who studied the fate of M_1 throughout the ageing period of Cheddar cheese, and noticed a concentration effect of M_1 to a much greater extent than we found with our Gouda cheese. Brackett mentioned several possible reasons to explain the effect. One is that proteolysis and lipolysis, which occur during the ageing process, could result in changes within the cheese, which allow recovery of more or less toxin in the analysis. If aflatoxin M_1 is bound to casein , as is suggested, cleaving of proteins or an increase in free fatty acids could act to change this binding. It can be assumed that aflatoxin M_1 is not covalently bound, since it can be extracted. One way M_1 could be bound is by hydrophobic interactions

with hydrophobic areas of the caseins. If this is true, one could explain how binding might be affected by the ageing or processing of Cheddar cheese. Proteolysis could cleave proteins in a way that allows less hydrophobic areas of the casein micelle to exist. Lipolysis results in the release of fatty acids which might, in turn, exert a detergent effect on the hydrophobically bound M_1. This might allow the toxin to be more readily retrieved by extraction.

Although there are differences in the results of the various studies on the effect of processing on M_1, they tend to come to the same conclusion: processing of milk does not destroy aflatoxin M_1, an observation which is of considerable practical importance.

METHODS OF ANALYSIS

Knowing now something about the background of the aflatoxin M_1 problem we can encage the analytical problem, the actual subject of this presentation. How can we detect and determine aflatoxin M_1 in milk and dairy products at concentrations of sub-micrograms per kilogram?

In general, there are two appraches possible for the detection and determination of mycotoxins: biological and chemical (Van Egmond, 1984a). Generally, chemical assays are to be preferred, because these generally are much more specific, more rapid, more reproducible and posses lower limits of detection. Because aflatoxin M_1 generally occurs in milk and

milk produots at very low levels, biological methods do not deserve further consideration.

The chemical assays that are used in aflatoxin M_1 methodology contain the basic steps of aech myootoxin analysis procedure (fig. 5). Sampling and sample preparation are not problems, because aflatoxin M_1 is homogeneously distributed in the milk or the milk produot. The extraction and clean-up however need much attention. Aflatoxin M_1 is a semipolar component, usually extracted with chloroform. Lipids and other substances that are co-extracted and which may interfere in the final detection, can be partly removed by liquid-liquid partitioning or by column chromotography for which often SiO_2 as a column packing material is used. Recently, also disposable commercially available prepacked columns have been incorporated in clean-up procedures, such as the C_{18} SEP paks (Fremy, 1981; Ferguson-Foos, 1984). This small cartridge is prepacked with a non polar phase, i.e. octadecylsilane bonded silica which allows direct application of the (diluted) milk on the artridge. Aflatoxin M_1 is trapped by the reversed phase packing material in the cartridge and can be eluted then by using solvents of decteased polarity.The direct application of milk onto the column avoids the problem of emulsion formation which may occur when milk (especially reconstituted milk from milkpowder) is extracted directly with chloroform in a separatory funnel. (The latter problem however can be largely overcome by addition of surfactants such as sodium dodecyl sulphate to the milk pior to shaking).

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After the clean-up procedure the extract must be concen-

trated to make detection of aflatoxin M_1 possible, as this toxin is usually present in very low amounts only. Despite the extraction and clean-up, the final extract may contain large amounts of other co-extracted substances possibly interfering with aflatoxin M_1 determination. Several possibilities exist to separate the mycotoxin from the matrix. Chromatographic procedures, which are physical separation techniques, are most often applied and they are used in combination with visual or instrumental determination of the amount of M_1. Immunoassays, which are bio-chemical separation techniques used in combination with instrumental determination are still in an early stage of development for mycotoxin research. Nevertheless these techniques are promising and some have been made suitable already for the determination of aflatoxin M_1. In this presentation attention shall be paid to thin layer chromatography, high performance liquid chromatography, radio immunoassay and enzyme linked immuno sorbent assay, as techniques used in the ultimate separation, detection and determination steps of modern methods of analysis for aflatoxin M_1.

Thin layer chromatography

In the first years of mycotoxin research, the early sixties, thin layer chromatography (TLC) became a very common and popular technique for separating extract components and nowadays it is a reliable, relatively simple and still the most frequently used technique for the determination of myco-

toxins. Initially, separations were carried out in one dimension using a single developing solvent. Later, two-dimensional thin layer chromatography was introduced to mycotoxin research (Kiermeier, 1970), a powerful separation technique in which a second development is carried out in a direction at right angles to the first one, using a different developing solvent. This provides a much better separation than one-dimensional TLC and is required especially in those cases where low levels of detection are necessary e.g. aflatoxin M_1 in milk. When TLC is applied, usually a final extract of milk (product) in chloroform is obtained.

Depending on the desired limit of detection, one- or two-dimensional TLC is applied. In Western European countries there is a tendency to establish tolerances for M_1 at very low levels, 0.1 g/kg or even less. In such cases, two-dimensional TLC should be the method of choice. Two-dimensional TLC is carried out as follows (see fig. 6): an aliquot of the sample extract is spotted at A and known amounts of aflatoxin M_1 standard are spotted at B. The plate is then developed in directions 1 and 2, respectively, using two different solvent systems. After development and drying of the plate, detection and quantification are carried out under longwave U.V.-light, thereby making use of the fortunate characteristic that aflatoxins emit the energy of absorbed longwave U.V.-light as fluorescent light. Figure 7 illustrates the separation pattern of an extract of milk, prepared according to the method of Stubblefield (1979), after two-dimensional TLC separation. The milk sample was contaminated at a

level of 0.1 g/kg. Quantifying of aflatoxin M_1 in the extract can be carried out by visual or densitometric comparison of the intensities of fluorescence of the M_1 spots from sample and standards. When two-dimensional TLC is applied, the method of Stubblefield (1979) has a limit of detection of ca. 0.01 g/l of milk. (This limit of detection relates to the lowest amount of M_1 visible on the TLC plate when exposed to U.V.-light of an intensity of $7mW/cm^2$. Although detectable at such a low level, aflatoxin M_1 can only be quantitated with a certain reliability by experienced analysis at level 0.03 g/l of milk (limit of quantitation).

In spite of the elaborate clean-up and separation techniques, interfering compounds, which have similar fluorescent and chromatographic behaviour as aflatoxin M_1, exist. In order to minimize the possibility of false-positives, the identity of aflatoxin M_1 in positive samples has to be confirmed. The most reliable method for this purpose is high resolutions mass spectroscopy (MS). MS, in combination with thin layer chromatography, however, is rather time consuming, and not every laboratory is equipped with this sophisticated type of apparatus. Therefore, simple chemical techniques are preferred. These techniques do not offer the same absolute certainty as MS, but they practically exclude false-positives. An example of such a confirmatory test is a method developed by Trucksess (1976) and modified by Van Egmond (1978). In that procedure, the presumed M_1 spot from the extract, as well as one of the standard spots (developed in direction 2), are derivatized on the TLC plate by superimposing a drop of tri-

fluoroacetic acid-hexane on each of these spots after two-dimensional TLC, followed by heating the plate at 75°C to allow the reaction to occur. The exact chemical nature of the reaction product which is formed is not yet known. If the reaction is carried out in test tube, the M_1 hemiacetal is formed, resulting from the acid-catalyzed addition of water to the terminal furan ring (fig. 8). However, on the TLC plate, another reaction occurs as the main product; it has a different R_f value to the M_1 hemiacetal. Like the M_1 hemiacetal, the M_1 derivative formed on the TLC plate has strong fluorescent properties, as well as a specific lower R_f value than M_1, and use is made of the latter characteristic in the confirmatory test. After the reaction, the plate is developed once more, the M_1 derivative from the sample and the standard are examined to determine if they have moved the same distance and show the same colour of fluorescence. The result of this test can be seen in figure 9.

The use of thin layer chromatography as a technique to separate aflatoxin M_1 from matrix components has decreased in recent years in favour of high performance liquid chromatography, and probably will further decrease in the near future in favour of the immunoassays. Nevertheless thin layer chromatography is still a major separation technique for the determination of aflatoxin M_1, especially its two-dimensional application which offers a good resolution, and consequently low limits of detection. Special advantages of thin-layer chromatography are the possibility of carrying out <u>in situ</u> derivatisation procedures to confirm the presence of myco-

toxins, the ability to store plates for later interpretation and the fact that the analyst has a certain "contact" with the results of the separation, because the human eye itself can act as a detector. Thin layer chromatography is particularly recommended to those who are inexperienced in the analysis of milk and milk products for aflatoxin M_1, and who cannot afford to purchase sophisticated analytical instrumentation.

High performance liquid chromatography

High Performance Liquid Chromatography (HPLC) or High Pressure Liquid Chromatography as it was initially called, became available for the analysis of foodstuffs in the early seventies and probably the first published application for mycotoxin research dates from 1973 (Seiber, 1973). After a somewhat hesitating start, the technique became of rapidly growing importance in the determination of mycotoxins, particularly when several types of column packings and (fluorescence-) detectors became available. The introduction of autosamples and computerized data retrieval systems made HPLC in principle very useful for large scale analyses. HPLC methods have also become available for the analysis of milk and milk products for aflatoxin M_1 (Glancy, 1978; Fremy, 1981; Tuinstra, 1982; Ferguson-Foos, 1984). Most of these methods make use of reversed phase columns, which contain bonded phase packings, e.g. octadecylsilane (ODS or C_{18}) bonded to the

silica support.

Solvents used in reverse phase systems are aqueous which means that the solvents used for preparation of the final extracts and the standard solutions to be injected also must be aqueous. Whereas in TLC the type of SiO_2 plate does not seem to be too critical for obtaining good results, the more sophisticated HPLC technique is more sensitive to variations in column types. There exist many similarly labelled reversed phase HPLC columns of good quality, which may have quite different chromatographic characteristics, due to both differences in the silica material used as a support and the technique used to form the bonded phase (Goldberg, 1982). Differences between various C_{18} columns when applied for the separation of M_1 containing milk extracts have been clearly demonstrated in a recent Dutch comparative study (Van Egmond, 1984): in figure 10 the results of HPLC separations are shown obtained by two laboratories that analysed the same sample with the same procedure, but they used different C_{18} columns and different fluorescence detectors. The differences are striking and the example clearly illustrates the importance of having available the right combination of column and detector.

In addition to the common HPLC procedures where aflatoxin M_1 is detected in reversed phase systems right away after separation of extract components, recently a method has been developed (Ferguson-Foos, 1984), in which aflatoxin M_1 is derivatized prior to HPLC separation and detection. The derivatization is achieved through reaction with trifluoroacetic acid (TFA) in the vial containing the final extract (or the

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M_1 standard). The reactionproduct has more polar properties than the parent compound, leading to a shorter retentiontime than aflatoxin M_1 in reversed phase systems. The thus obtained change in elution pattern can be beneficial, especially where the aflatoxin M_1 peak does not fully separate from the peaks of luorescent extract components. An additional advantage of the derivatization is the increase in fluorescence intensity of the M_1 derivative compared to the parent compound, allowing lower limits of detection. An example of a high performance liquid chromatogram thus obtained from milkpowder reconstituted to milk, and contaminated with M_1 at a level of ca. 0.03 g/l (which is a commonly found level of contamination in western Europe) is given in figure 11. It may be noticed that the separation of the peak of interest is not yet complete and that further optimalisation is required. The method of Ferguson-Foos (1984) has been studied collaboratively in 1983 but the study has to be stopped halfway when collaborators encountered problems in dissolving derivatized M_1 solvents in the HPLC solvent. The problems were traced back to irreversible and inconsistent adsorption of the derivative to the glasswall of the vials and have recently been overcome by sililation of the vials rpior to the derivatization reaction which leads to inactivation of the glasswall so that adsorption is largely prevented. It is anticipated that the collaborative study will be re-initiated this year.

High performance liquid chromatography has partly superceded thin layer chromatography in the analysis of food for mycotoxins. The reasons for this development are obvious.

Separations can be accomplished in a matter of minutes, HPLC methods generally provide good quantitative information and the equipment employed in HPLC systems can be automated rather easily. Finally, on-line coupling with a mass spectrometer has become possible, although it is too early to estimate the value of the latter technique for the determination and confirmation of mycotoxins.

HPLC has limitations as well. Although resolutions are much better than those obtained using one-dimensional TLC the use of two-dimensional chromotography in HPLC is hardly possible. It is just the latter technique that has proven to be such a powerful separation tool when applied to thin layer chromatography, especially when low limits of detection are required. The cost of equipment for thin layer chromatography is relatively cheap (except densitometers) compared with the expensive instrumentation for HPLC. The extensive experience required to obtain the maximum benefit from an HPLC system contitutes another limitation, whereas TLC can be learned relatively easily.

The limits of detection and determination of HPLC and TLC systems are comparable when milkextracts are analysed for aflatoxin M_1. The variability of repetitive injections in HPLC may be slightly lower than that of repetitive spottings in TLC. However this difference is of minor importance as the error in the ultimate separation and detection step contributes only for a smaller part to the total error in the whole analysis procedure. As yet no publications have appeared on comparative studies of HPLC and TLC techniques, when applied

for the determination of aflatoxin M_1 in the same samples of milk. The provisional results of a Dutch comparative study (Van Egmond, 1984) however, indicated that both techniques provide results that agree rather well. In conclusion HPLC can be an attractive alternative to TLC in the determination of aflatoxin M_1 in milk(products). However one should bear in mind that sophisticated systems are weak if the availability of supplies spare parts and service form a problem, as may be the case in many of the developing countries. Especially in the latter situation HPLC should not be the first choice when setting up a system for monitoring or surveying milk(products) for aflatoxin M_1.

Immunochemical procedures

Immunochemical procedures are based upon quite different principles than chromatographic procedures. An immunoassay is an assay in which the molecular recognition properties of antibodies, bio-macromolecules are used, rather as a lock responds to a key. The key to be measured is the antigen (in our case aflatoxin M_1), which can be labelled for the purpose of counting.This lavel can be a radioisotope, an enzyme, a fluorescent group or some other marker which can easily be quantified. For the determination of mycotoxins the use of immunoassays has been limited to date to radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA).

It is beyond the scope of this presentation to discuss the production of antibodies against mycotoxins, which gene-

rally have molecular weights too low to evoke antibodies directly when administered to animals. These so-called haptens have to be conjugated with proteins before immunisation can occur. Up to now it has been possible to evoke antibodies against several of the mycotoxins, aflatoxin M_1 included. For the performance of immunoassays it is further necessary to have available radiolabelled mycotoxins (for which often ^3H is used) in the case of RIA and enzyme-labelled mycotoxins for ELISA (for which often horseradish peroxidase is used). The extraction and clean-up procedures applied in immunoassays developed for mycotoxins are generally the same as those for the chromatographic proceduresm though because of the high specificity of immunoassays crude extracts are sometimes suitable. The final extracts used in immunoassay are (buffered) aueous solutions.

In figure 12 the mechanism of a radioimmunoassay is outlined. The test portion, containing a known amount of labelled antigen (marked as active) and an unknown amount of unlabelled antigen (the mycotoxin looked for, i.e. aflatoxin M_1) is brought into contact with a fixed amount of antibody. Competition takes place between labelled and unlabelled antigen for the active sites of the antibody. After a certain time equilibrium is reached, and there will remain some free antigen, the rest is bound to the antibody. The relative binding rationof the labelled and unlabelled antigen to the antobody depends on the relative concentration ratio of the labelled and unlabelled antigen in the test portion. The lower the ratio labelled antigen/unlabelled antigen, the lower the

radioactivity of the antigen-antibody complex. After separation of the antigen-antibody complex and the free fraction the radioactivity of the complex is measured in a liquid scintillation counter. This radioactivity is a measure of the amount of unlabelled antogen (the mycotoxin look for) in the test portion. Normally the evaluation of the amount of mycotoxin in an unknown sample is made by using a standard curve.

Harder (1979) introduced a RIA procedure for the determination of aflatoxin M_1 in milk. The method has a limit of detection reported to be at ca. 0.25 g M_1/litre milk, a limit that cannot yet compete with those obtainable with chromatographic procedures, which are about one order of magnitude lower.

In the application of enzyme-linked immunosorbent assay in mycotoxin research several variants exist, one of which is the competitive assay applied for the determination of aflatoxin M_1 in milk (Pestka, 1981) (fig. 13). A polystyrene tube or microtitre plate is coated with a known amount of antibody against aflatoxin M_1. After being washed, the test solution containing an unknown quantity of aflatoxin M_1 is added, directly followed by the addition of a known quantity of aflatoxin M_1, labelled with enzyme. Labelled and non-labelled aflatoxin M_1 compete for the active sites of the bound antibody. After incubation the tube or plate is washed again and the captured enzyme is determined by adding chromogenic substrate. The resulting colour can be measured instrumentally or visually. The lower the concentration of reaction product,

the lower the amount of bound enzyme and the higher the aflatoxin M_1 concentration in the test portion. As with RIA a standard curve is established from which the toxin concentration in the test portion is then determined. The limit of detection of the ELISA procedure of Pestka (1981) was reported to be at 0.25 g/l of milk which is comparable with the performance of the RIA procedure and about one order of magnitude higher than detectionlimits for aflatoxin M_1 obtained with chromatographic procedures.

The application of immunoassays for the determination of mycotoxins is a recent development, so that collaborative studies on this subjecthave not yet been conducted. The present lack of method performance characteristics derived from collabotative studies makes it difficult to estimate the value of the newcomers compared to HPLC and TLC. Incidentally RIA and ELISA have been compared to each other for the determination of aflatoxin M_1 in milk. It was concluded that ELISA was the preferred method mainly because of its simplicity, sensitivity and selectivity. Taking into account the disadvantages of RIA, such as the limited shelf-life activity of the radioisotopes, problems of radioactive waste disposal or licensing requirements and the need of an expensive scintillation counter, it is to be expected that ELISA in particular will be of growing importance as an assay technique for mycotoxins, aflatoxin M_1 included. However, a matter of continuous vigilance is the specificity of immunoassays. Although most of the antisera produced against mycotoxins seem to be quite specific, the possibility of crossreactions cannot be fully

ruled out and it is a good laboratory practice to confirm positive findings of immunoassays by methods of analysis based on other principles.

CONCLUSION

Summarizing the present state of methodology for the determination of aflatoxin M_1 in milk and milk products it can be concluded that

1) Thin layer chromatography, although a veteran in mycotoxin methodology is a reliable, feasible and relatively simple separation technique. Its two-dimensional application offers especially good resolution, resulting in low limits of detection, which are usually needed when milk is analysed for aflatoxin M_1.

2) High performance liquid chromatography can be an attractive alternative to thin layer chromatography. This more expensive technique offers the possibility of automating the ultimate separation and quantification steps. However, application of the technique requires advanced skill, facilities and service, which makes the technique vulnerable in suboptimal circumstances.

3) Immunoassays such as radioimmunoassay and enzyme-linked immunosorbent assay are promising techniques. Although still in its infancy, it is expected that especially ELISA will play an important role in aflatoxin M_1 methodology in the near future and that simple "field kits" will allow the application of the new technique also in the developing countries.

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Legend to figures:

Figure 1 Chemical structures of aflatoxins B_1 and M_1

Figure 2 Frequency distribution of M_1 amounts in samples of Dutch consumption milk (1981)

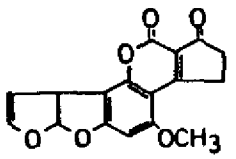
Figure 3 Frequency distribution of M_1 amounts in samples of Dutch consumption milk (1972)

Figure 4 The effect of processing on the aflatoxin M_1 content of milk and milk products

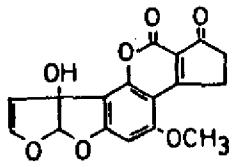
Figure 5 Analytical procedure for mycotoxin determination

Figure 6 Schematic of thin layer chromatogram for two-dimensional chromatography. Direction 1: diethylether-methanol-water (94+4.5+1.5, lined tank). Direction 2: chloroform-acetone-methanol (90+10+2, unlined tank). Measures are in centimetres.

Figure 7 Two-dimensional separation of milkextract contaminated with aflatoxin M_1 (0.1 g/l), prepared according to the method of Stubblefield (1979)



aflatoxin B_1



aflatoxin M_1

Figure 1

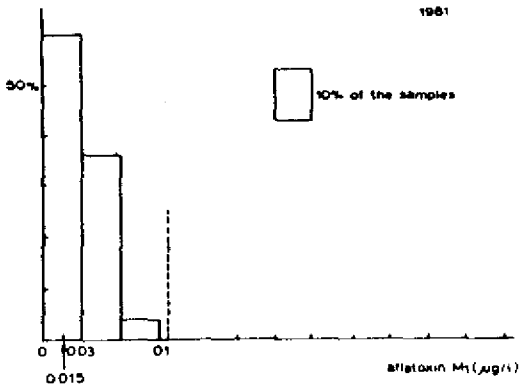
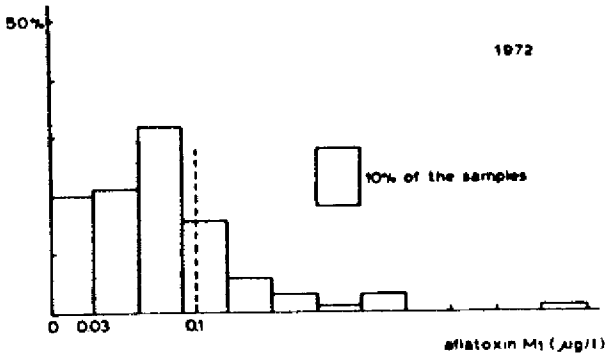


Figure 2



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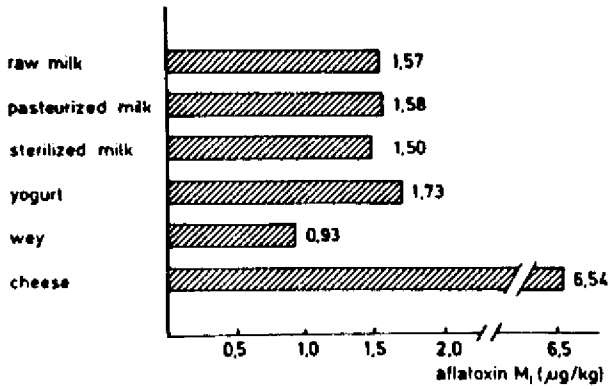
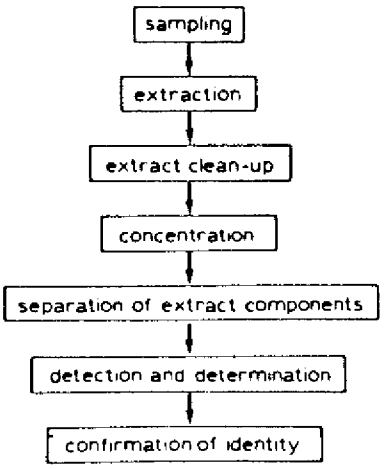


Figure 4

Figure 5



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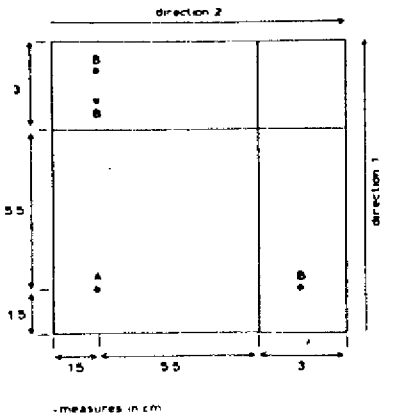


Figure 7

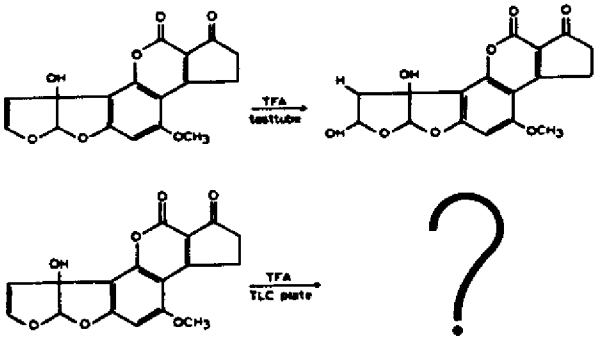
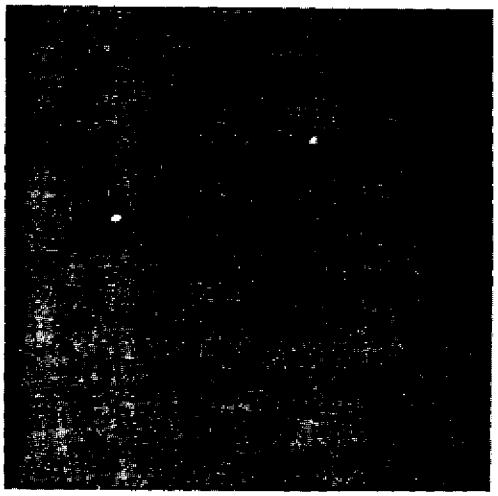


Figure 8



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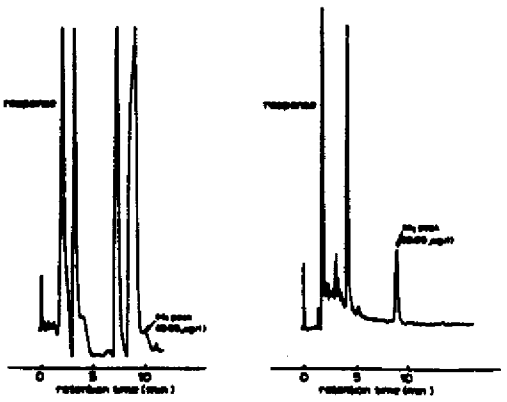
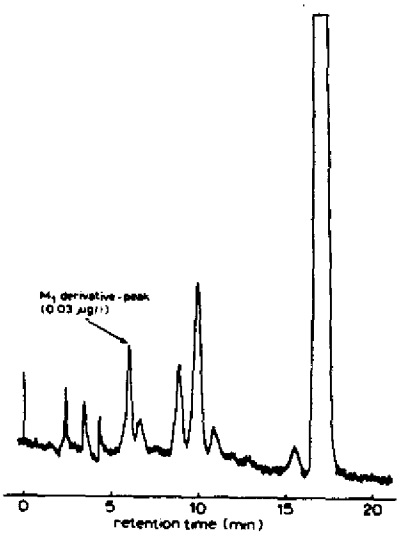


Figure 10

Figure 11



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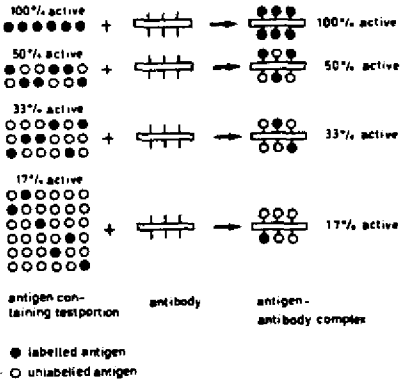

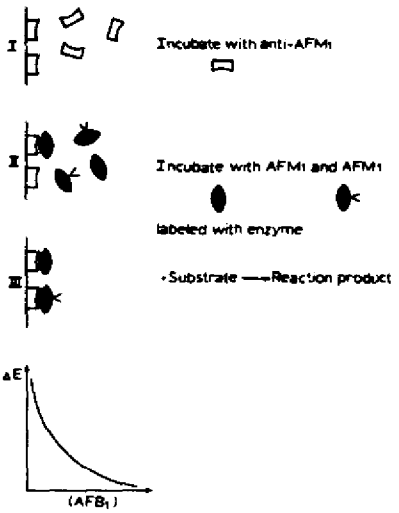

Figure 13

Зак.3516 ПИК ВИНИТИ