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Determination of aflatoxin M1 using a dialysis-based immunoaffinity sample pretreatment system coupled on-line to liquid chromatography

Reusable immunoaffinity columns

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ABSTRACT

A liquid chromatographic column-switching system containing a dialysis unit and an anti-aflatoxin immunoaffinity precolumn (immuno precolumn) is described for the automated determination of aflatoxin M1 in milk samples. Both a flat membrane dialysis unit working according to the flowing donor–flowing acceptor principle and a laboratory made hollow-fibre dialysis unit working according to the stagnant donor–flowing acceptor principle were evaluated. The hollow-fibre unit is superior with respect to repeatability (3% relative standard deviation) and detection limit (10 ng/l for aflatoxin M1 in milk), in spite of the fact that the overall recovery is only 6%. Interfering compounds, which would destroy the activity of the immuno precolumn, are efficiently removed from the system by the dialysis step; a single immuno precolumn can then be used for over 70 milk analyses. No decrease in the performance of either the immuno precolumn or the hollow-fibre dialysis unit is observed.

INTRODUCTION

Because of the complex sample matrix and the low detection limits (10–50 ng/l) required, the liquid chromatographic (LC) determination of aflatoxin M1 in milk and milk products usually needs an extensive sample clean-up [1,2]. Recently, immunoaffinity columns containing immobilized antibodies directed against aflatoxins have become commercially available. With these immuno columns, the whole sample pretreatment procedure is reduced to a one-step solid-phase extraction [3–6]. Owing to the selective antibody–antigen interaction, the aflatoxins immunosorbent packed into a stainless-steel precolumn can be incorporated into an automated column-switching system, allowing fully automated sample pretreatment and analysis [5,6]. Unfortunately, when analysing milk samples, these immuno precolumns cannot be reused. In contrast to aqueous standards, with milk samples a continuous decrease in the capacity is observed. Proteolytic enzymes present in milk probably cause the degradation of the immobilized antibodies. This assumption seems likely because, with an anti-oestrogen immuno precolumn, a decrease in immuno precolumn capacity was observed if protease-containing serum samples were analysed [7]. Heating of the milk samples was not extensively studied because this is a laborious off-line procedure and because during the initial experiments coprecipitation of the aflatoxins with the fatty material occurred.

As an immunoaffinity precolumn is relatively expensive, its reuse would significantly reduce the cost of analysis. With this aim in mind, on-line removal

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of the interfering milk components by means of dialysis, prior to immunoaffinity sample pretreatment, was studied in this work with a flat membrane dialyser and a hollow-fibre dialyser.

EXPERIMENTAL

Chemicals

Aflatoxin B2 was a gift from RIKILT (Wageningen, Netherlands) and aflatoxin M1 from the National Institute of Public Health and Environmental Protection (Bilthoven, Netherlands). Aflatoxins M2, P1 and Q1 were obtained from Sigma (St. Louis, MO, USA). LC-grade acetonitrile and methanol were purchased from Baker (Deventer, Netherlands). LC-grade water was prepared from demineralized water using a Milli-Q water purification system (Millipore, Bedford, MA, USA) with subsequent filtration through a 500 mm × 10 mm I.D. LC column filled with 40-μm Baker C_{18} bonded phase. All other chemicals were of analytical-reagent grade.

The LC eluents were degassed under vacuum in an ultrasonic bath. Plastic precolumns containing the immobilized monoclonal anti-aflatoxin antibodies were obtained as a gift from Koopman & Koek (Rijswijk, Netherlands). Unfortunately, it is not known exactly against which aflatoxins the antibodies were raised, and no information about the cross-reactivity is known. The precolumns were opened and the immunosorbent was removed and repacked in the stainless-steel precolumns as described previously [8]. When not in use, the immuno precolumns were stored in methanol–water (70:30, v/v).

Apparatus

An Amicon (Danvers, IL, USA) dialyser with a 15 000-dalton molecular-weight cut-off cellulose acetate membrane, with a 1.3-cm^2 accessible membrane area and with acceptor and donor channel lengths of 65 cm each and acceptor and donor volumes of 0.8 ml each, was used as the flat membrane dialysis unit.

The hollow-fibre membrane dialysis unit was constructed from 15 000-dalton molecular-weight cut-off cellulose acetate-based dialysis fibre which were cut from an Organon (Turnhout, Belgium) Nephross Allegro HF artificial kidney. Each fibre had an I.D. of 200 μm and a membrane thickness of 6.5 μm, and 105 fibers were glued into two fittings with Bindulin (Fürth, Germany) silicone glue. The average fibre length accessible for dialysis was about 10 cm. For use, the dialysis unit was inserted into a 25- or 30-ml sample vial which was equipped with a magnetic stirrer. When not in use, the unit was stored in a solution containing 0.02% sodium azide in LC-grade water.

The set-up of the LC system used for flat membrane dialysis with immunoaffinity preconcentration is shown schematically in Fig. 1. It consisted of two Gilson (Villiers-le-Bel, France) Model 302 pumps [one for the separation column (pump 4) and one for the water dilution step (pump 3)], two

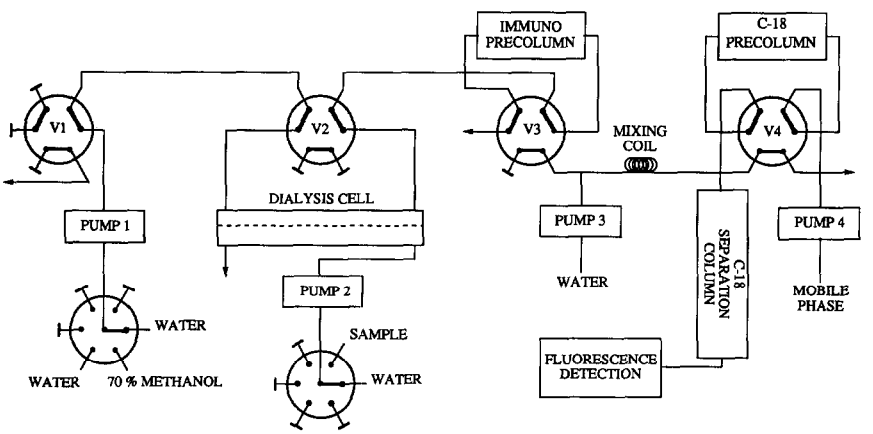

Fig. 1. Set-up of the total analytical system used for on-line dialysis/immuno preconcentration with the flat membrane dialysis unit.

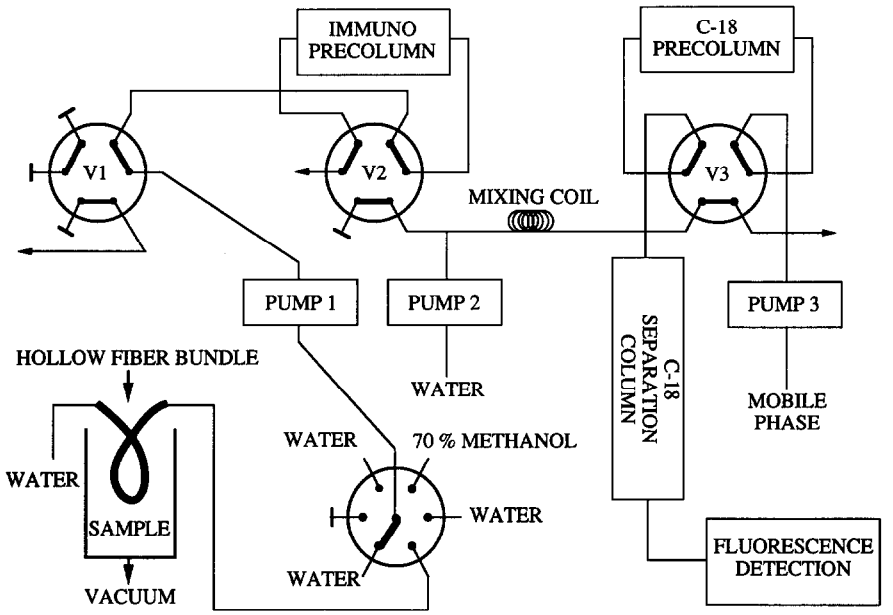

Fig. 2. Set-up of the total analytical system used for on-line dialysis/immuno preconcentration with the hollow-fibre membrane dialysis unit.

Applied Biosystems (Ramsey, NJ, USA) Spectroflow 400 pumps (pumps 1 and 2), a Spark (Emmen, Netherlands) Prospect 1.0 programmer containing the valve-switching units and the solvent-selection valves, a Perkin-Elmer (Beaconsfield, UK) LS-4 fluorescence detector (λ_{ex} = 364 nm, λ_{em} = 434 nm for aflatoxin M1; λ_{ex} = 365 nm, λ_{em} = 440 nm for all other aflatoxins) and a Kipp & Zonen (Delft, Netherlands) BD 40 recorder. The separation column was a laboratory-packed 100 mm × 3.0 mm I.D. or 200 mm × 3.0 mm I.D. glass column with 5-μm LiChrosorb RP-18 (Merck, Darmstadt, Germany), protected with a 10 mm × 2.0 mm I.D. guard column packed with the same material. The 20 mm × 3.0 mm I.D. stainless-steel C_{18} precolumn was packed with 40-μm Baker C_{18} bonded particles. The mixing coil was a stainless-steel LC capillary with an I.D. of 1.1 mm and an internal volume of 1 ml. Pumps 1 and 2 and all switching valves were computer controlled.

The set-up of the LC system used for hollow-fibre dialysis with immunoaffinity preconcentration is shown schematically in Fig. 2. It consisted of essentially the same components as the flat membrane dialysis system. The sample vial of the hollow-fibre unit was connected to an additional valve and a vacuum supply, controlled by the computer, such that the contents of the vial could be removed automatically.

Separation was performed at ambient temperature using acetonitrile–methanol–water (16:24:60 or 20:5:75, v/v/v) as the mobile phase. The flow-rate was 0.5 ml/min. The immuno precolumns were laboratory-made 10 mm × 10 mm I.D. stainless-steel columns equipped with 5-mm stainless-steel screens and PTFE rings as the column inlet and outlet.

Analytical procedures

Hollow-fibre dialysis/immuno preconcentration was performed with the set-up in Fig. 2. The time schedule of the analytical procedure is given in Table I. After placing a sample in the sample vial and preconditioning the immuno precolumn with 10 ml of water using pump 1, dialysis was started. During this process, water (as the acceptor phase) was sucked through the hollow-fibre bundle with pump 1 and subsequently pumped over the immuno precolumn for preconcentration of the analytes. Then the immuno precolumn was flushed with 5 ml of water (pump 1) to displace the remaining sample and to remove non-specifically bound impurities. Simultaneously, the C_{18} precolumn was precondi-

TABLE I

SCHEDULE OF THE ON-LINE ANALYTICAL PROCEDURE USING THE SET-UP ACCORDING TO FIG. 1

Step	Event	Valve positions[a]		
		Valve 1	Valve 2	Valve 3
1	Flushing immuno precolumn with water (20 min, 5.2 ml)	A	A	A
2	Flushing capillaries with sample	B	A	A
3	Flushing immuno precolumn with sample (4 min, 10.4 ml)	A	A	A
4	Flushing capillaries with water	B	A	A
5	Flushing immuno precolumn with water via pump 1 (2 min, 5.2 ml) and simultaneously flushing C_{18} precolumn with water via pump 2 (1 min, 4.7 ml)	A	A	B
6	Flushing capillaries with methanol–water (70:30, v/v)	B	A	B
7	Analyte desorption from immuno precolumn with methanol–water (70:30, v/v) (2 min, 0.52 ml) with subsequent dilution with water (2 min, 9.4 ml) and analyte reconcentration of the C_{18} precolumn	A	B	B
8	Desorbing C_{18} precolumn by switching on-line with separation column and flushing immuno precolumn with additional methanol–water (70:30, v/v) (0.25 min, 0.65 ml)	A	B	A
9	Flushing capillaries with water	B	A	A

[a] Position A corresponds with the valve positions shown in Fig. 1.

tioned with 5 ml of water via pump 2. Subsequently, the immuno precolumn and the C_{18} precolumn were switched in series and the immuno precolumn was desorbed with methanol–water (70:30, v/v) in the backflush mode using pump 1. The methanol–water eluate was diluted with water to a methanol content of 4% by pump 2 in order to allow preconcentration of the aflatoxins on the C_{18} precolumn. After transfer of the analytes to this precolumn, the actual separation was started by switching the C_{18} precolumn on-line with the separation column, using pump 3 for the eluent. The immuno precolumn was flushed with an additional 0.65 ml of methanol–water (70:30, v/v). Next the hollow-fibre bundle was purged with water via pump 1. At the same time the sample was removed from the vial by vacuum action and the vial and the outside of the hollow-fibre bundle were manually purged with about 100 ml of water. Finally, the sample vial was filled with the next sample, or with a sodium azide solution, if no further analysis had to be performed.

For hollow-fibre dialysis/C_{18} preconcentration the set-up according to Fig. 2 was modified such that the immuno precolumn was omitted, while V1 was directly connected to V3, that is, the acceptor stream was directly preconcentrated on the C_{18} precolumn.

For flat membrane dialysis/immuno preconcentration, the set-up according to Fig. 1 was used. The analytical procedure was the same as with hollow-fibre dialysis with immuno preconcentration, except that in place of a stagnant donor a flowing donor was used. Before analysis the capillary connecting pump 2 and the dialysis cell was disconnected from the donor inlet, purged until it was completely filled with sample and connected to the donor inlet again. This was done in order to reduce mixing effects.

For flat membrane dialysis/C_{18} preconcentration, the set-up according to Fig. 1 was modified such that the immuno precolumn was omitted, while V2 was directly connected to V4, that is, the acceptor stream was directly preconcentrated on the C_{18} precolumn.

The analytical procedures for direct preconcentration of samples on the C_{18} precolumn or on the immuno precolumn, without dialysis, have been described elsewhere [5,6].

Samples

Milk samples were freshly bought each morning, stored at 4°C and used during the same day. The samples were brought to room temperature on a water-bath 30 min before analysis; subsequently they were spiked and analysed without further pretreatment. For spiked milk samples the recovery was corrected by subtracting the amount of aflatoxin M1 found in the blank. The amount of analyte determined was calculated by peak-area comparison with a standard loop injection. As expected, blank values for aflatoxin M1 in milk were found to be 10–30 ng/ml. The identity of the aflatoxin M1 peak in the blank was confirmed by comparison of the retention time and the excitation and emission maxima with those for an aqueous standard.

The dialysis efficiency was determined as the ratio of the peak area found with dialysis to that found by direct preconcentration of the same sample, using a volume equal to the acceptor volume in the dialysis experiment (for explanation, see below).

RESULTS AND DISCUSSION

The 10 mm × 10 mm I.D. immuno precolumn allowed the preconcentration of aflatoxin M1 from aqueous standard solutions up to a volume of 30 ml with a constant recovery of about 70% (see also ref. 5). If larger volumes were preconcentrated, the recovery decreased owing to breakthrough. This implies that, for an accurate and precise performance of the final analytical procedure, the acceptor volume from the dialysis unit, which has to be preconcentrated on the immuno precolumn, should not exceed 30 ml. Consequently, the detection limit of the method will depend only on the analyte concentration which can be achieved in the acceptor phase. The ratio between the analyte concentration in this phase and the original analyte concentration in the sample (termed the dialysis efficiency in this paper) will therefore characterize the performance of the dialysis process most appropriately. If the donor and acceptor volumes are equal, the dialysis efficiency can be, at best, 0.5. However, if the donor volume is much larger than the acceptor volume, in theory a dialysis efficiency of 1.0 can be obtained. One main aim in the following experiments was to optimize the on-line dialysis processes with respect to the dialysis efficiency.

Owing to the limited availability and high cost of aflatoxin M1, the initial characterization of the dialysis units was performed with aflatoxin B2. Although the chemical structure of aflatoxin M1 is related more to aflatoxin B1 than to aflatoxin B2, the latter was used because of its higher intrinsic fluorescence sensitivity and the fact that aflatoxin B1 is toxic. Because of the much shorter analysis time, initial optimization and evaluation of on-line dialysis were performed with subsequent preconcentration on a C_{18} precolumn.

Flat membrane dialyser

Amongst the various donor–acceptor dialysis configurations (stagnant–stagnant, stagnant–flowing, flowing–stagnant and flowing–flowing), the last alternative was selected, because the expected donor and acceptor volumes far exceeded the volumes of the donor and acceptor chamber (0.8 ml each) of the unit.

Influence of dialysis time on recovery. A standard solution containing 80 ng/l of aflatoxin B2 was analysed with flat membrane dialysis and C_{18} preconcentration. The donor and acceptor flow-rates were kept constant in all experiments at 0.5 and 0.52 ml/min, respectively, using the counter-current mode. According to theory, the recovery should be constant, irrespective of the duration ot the dialysis process. This was found to be true for dialysis times of at least 10 min. As a 10-min dialysis time gave a yield of 80–90% of that obtained under steady-state conditions, this time was chosen to characterize the dialysis unit in the subsequent experiments.

Influence of donor flow-rate on dialysis efficiency. With a constant dialysis time of 10 min and a constant acceptor flow-rate of 0.52 ml/min, *i.e.*, with an acceptor volume of 5.2 ml, experiments were performed using a donor flow-rate of 0.5, 1.0 or 2.0 ml/min, *i.e.*, with donor volumes of 5.0, 10 and 20 ml, respectively. Over this range a virtual constant dialysis efficiency of 0.1 ± 0.005 was found. One can therefore expect that a further increase in the donor flow will not significantly improve the efficiency.

Influence of acceptor flow-rate on dialysis efficiency. With the same dialysis time of 10 min and a constant donor flow-rate of 0.5 ml/min (total donor volume 5 ml), the acceptor flow-rate was varied as 0.26, 0.52 and 1.3 ml/min (volumes of 2.6–13 ml).

Under these conditions, the dialysis efficiency decreased (0.11, 0.09 and 0.05, respectively). Although a further decrease in the acceptor flow-rate will certainly improve the efficiency further, such an approach is highly impractical because of the long dialysis times required for what is intended to be an on-line method.

On-line combination of dialysis and immuno preconcentration. On the basis of the above, as a compromise, a donor flow-rate of 0.65 ml/min and an acceptor flow-rate of 0.52 ml/min were chosen to combine dialysis and immunoaffinity preconcentration on-line. Of course, different flow-rates would also have been suitable; the present values were selected because of the available pump settings.

As a compromise between a short analysis time and low detection limits, a dialysis time of 20 min, corresponding to donor and acceptor volumes of 13 and of 10.6 ml, respectively, was chosen. Eleven milk samples spiked with 0–500 ng of aflatoxin M1 were analysed. The trend of a decreasing recovery with an increase in the number of analyses, clearly observed in a previous study in which no dialysis step was included in the procedure [6], was not found with the present system (*cf.*, Fig. 3). This indicates that constituents of the milk sample that adversely affect the activity of the immuno precolumn have indeed been removed. A drawback of the system is the poor reproducibility for aflatoxin M1 in spiked milk [60–100 ng/l; relative standard deviation (R.S.D.) = 12% (n = 9), possibly caused by air bubbles formed in the donor stream from time to time.

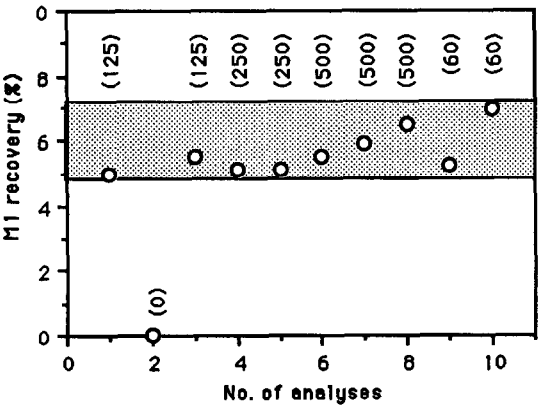

Fig. 3. Recovery as a function of number of analyses performed with the flat membrane dialysis/immunoaffinity preconcentration system. Crude milk samples were spiked with the indicated amounts of aflatoxin M1 and dialysed for 20 min, with donor and acceptor volumes of 13 and 10.6 ml, respectively.

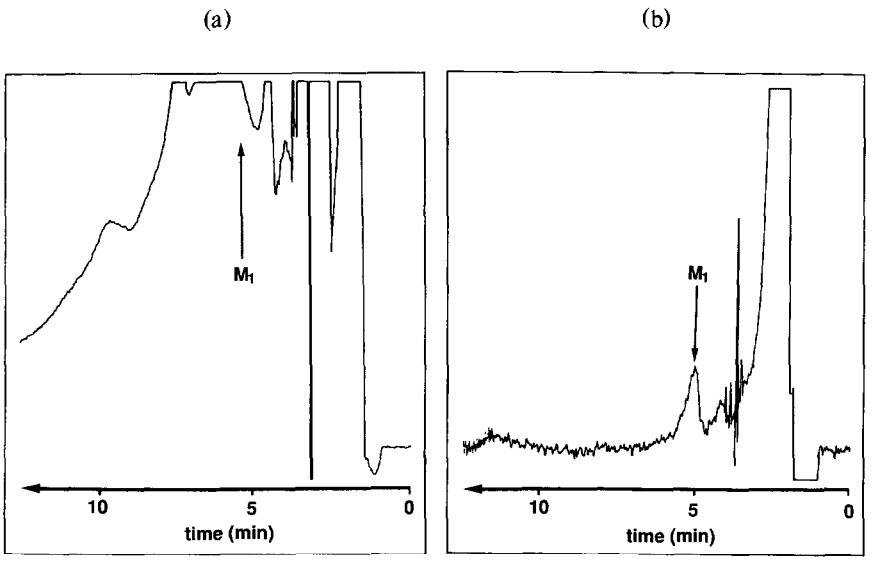

Fig. 4. LC of a non-spiked milk sample, analysed according to (a) the flat membrane dialysis/C_{18} preconcentration procedure or (b) the hollow-fibre dialysis/immunoaffinity preconcentration procedure. Donor and acceptor volumes: (a) 6.5 and 5.3 ml, respectively; (b) 26 and 21.5 ml, respectively, with the fluorescence detector set to a five times higher sensitivity.

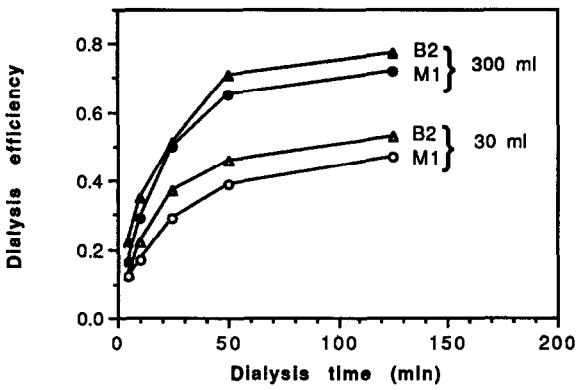

Fig. 5. Dialysis efficiency as a function of dialysis time. Acceptor volume, 25 ml. The dialysis time was varied between 5 and 125 min by adjusting the acceptor flow-rate. Plots were recorded for donor volumes of either 30 or 300 ml, containing either 30 ng/l of aflatoxin M1 or 4 ng/l of aflatoxin B2. The analyses were run according to the hollow-fibre dialysis/C_{18} preconcentration procedure. Dialysis efficiencies calculated as described under Experimental.

As was to be expected, combining dialysis with immunoaffinity sample pretreatment did not increase the selectivity of the system and similar chromatograms were obtained with and without dialysis. The high selectivity of the immunoaffinity step can be seen by comparing the chromatograms of the dialysis/immunoaffinity preconcentration and the dialysis/C_{18} preconcentration system (Fig. 4). In the latter instance, after dialysis, the sample was preconcentrated directly on a C_{18} precolumn. In both instances non-spiked crude milk samples were analysed. The aflatoxin M1 concentration in the sample was 25 ng/l. One should note the differences in sample volume and detector sensitivity with the two methods in Fig. 4. The bad peak shape for aflatoxin M1 in Fig. 4 can be explained by the poor performance of the analytical column.

Hollow-fibre dialysis unit

In order to improve the reproducibility and also the dialysis efficiency of the system, another dialysis unit was designed. The surface area of the dialysis membrane and its thickness are significant parameters which determine the speed of the dialysis process. Improvement of either parameter with the flat membrane dialyser is technically difficult. We therefore evaluated the use of hollow-fibre membranes, which are very stable and easy to handle, although they have a membrane thickness of only between 5 and 10 μm.

The construction and design of the hollow-fibre membrane dialyser are described under Experimental. The overall membrane area available for dialysis was calculated to be 60–70 cm^2, which is about 50 times larger than that with the flat membrane dialyser. Because of the hollow-fibre bundle design, stagnant donor-flowing acceptor dialysis, with the inner fibre volume flushed by the acceptor phase and the bundle inserted in the sample solution, seemed most appropriate for analysis.

Dialysis efficiency. The efficiency of the hollow-fibre dialysis unit was determined using the hollow-fibre dialysis/C_{18} preconcentration system (see Experimental). In Fig. 5 the dialysis efficiency is displayed as a function of the dialysis time. In all experiments the acceptor volume was held constant at 25 ml. The dialysis time was varied between 5 and 125 min by adjusting the acceptor flow-rate. All four plots show a steep initial rise, a plateau being reached for dialysis times of over about 50 min. This means that, for an on-line analytical system, dialysis times between 10 and 500 min are a good compromise between short analysis time and high recovery.

Fig. 5 also demonstrates that the dialysis efficiency depends on the sample volume (stagnant donor volume) used. Using, *e.g.*, a 300-ml instead of 30-ml donor volume, gives a dialysis efficiency of 0.65 instead of 0.39 for aflatoxin M1 and of 0.71 instead of 0.46 for aflatoxin B2 (dialysis time 50 min). Actually, with an infinitely large donor volume, the dialysis efficiency will reach a plateau value of 1.0.

It should be obvious that, with a larger donor volume, a higher concentration gradient between the donor and acceptor phases and hence a higher transfer rate of analytes can be achieved, that is, the amount of analytes transferred will be higher. Consequently, although the recovery will decrease, the detection limit will improve, as the absolute amount of analyte which is extracted from the increased sample volume will be larger.

Finally, from Fig. 5 one can read that a 30-ml donor volume dialysed for 20 min with a total acceptor volume of 25 ml will give a dialysis efficiency for both aflatoxins tested of about 0.3. This is three times better than with the flat membrane dialysis unit.

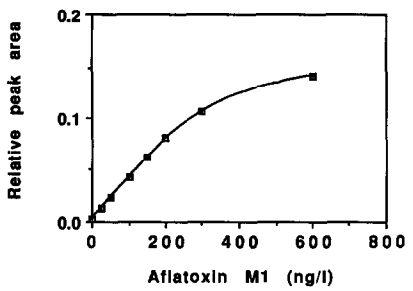

Fig. 6. Calibration graph recorded with milk samples spiked with aflatoxin M1. Crude untreated milk samples were analysed according to the hollow-fibre dialysis/immunoaffinity preconcentration procedure. Donor and acceptor volumes, 25 ml each.

On-line hollow-fibre dialysis/immuno preconcentration: analytical data

A donor volume of 25 ml was adopted, as a vial of this size was available which was suitable for magnetic stirring and had a proper fitting at the bottom, which could be used for automated removal of sample solutions via a vacuum supply.

Standard samples. A calibration graph was constructed using aqueous standard samples containing 10–300 ng of aflatoxin M1 (dialysis time 20 min; acceptor volume 25 ml). The graph was linear in this range (r = 0.9993; n = 6). The recovery found was 20% for aqueous samples.

Spiked milk samples. As with the aqueous solutions, spiked milk samples were analysed without any prior sample pretreatment. The calibration graph (Fig. 6) for aflatoxin M1 was linear (r = 0.9998; n = 6) up to 200 ng/l of analyte. The recovery with milk samples was, however, distinctly lower (6%; R.S.D. = 3%, n = 5) than with spiked water samples (20%). The lower recovery is probably due to analyte–protein interactions which occur in milk samples. Although this recovery is low, it is not a problem during the analysis of real samples because relatively large sample volumes can be processed with a repeatability of *ca.* 3%. Under the conditions used (25-ml samples), the detection limit for aflatoxin M1 in milk was 10 ng/l (signal-to-noise ratio = 3:1). Increasing the sample volume to 300 ml will improve the detection limit by almost a factor of two (*cf.*, Fig. 5). Another method to improve the detection limit, without increasing the analysis time, is to use a hollow-fibre bundle which contains more fibres. Actually, a bundle containing four times more fibres can still be placed in the present 25-ml sample vial without affecting the stirring efficiency of the sample.

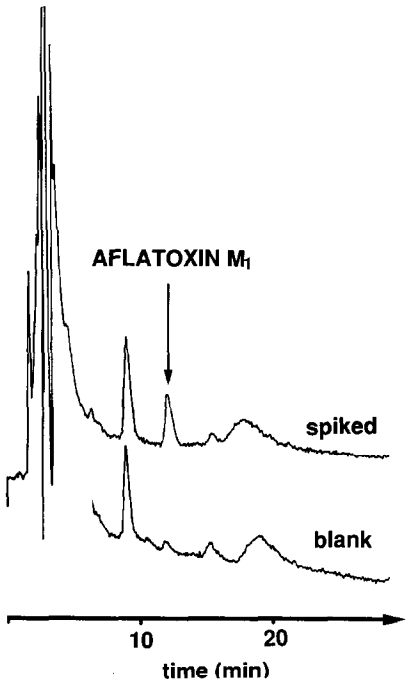

Fig. 7. LC of a crude milk sample spiked with 50 ng/l of aflatoxin M1, and the corresponding blank. Hollow-fibre dialysis/immunoaffinity preconcentration was performed with donor and acceptor volumes of 25 ml each. Mobile phase, acetonitrile–methanol–water (20:5:75, v/v/v). For further details, see Experimental.

Chromatograms of a crude milk sample spiked with 50 ng/l of aflatoxin M1 and of the corresponding blank are shown in Fig. 7. The hollow-fibre dialysis/immunoaffinity preconcentration was performed with donor and acceptor volumes each of 25 ml. The aflatoxin M1 level found in the blank sample corresponded to the detection limit of the method of 10 ng/l. With the initial mobile phase of acetonitrile–methanol–water (16:24:60, v/v/v) an interfering peak coincided with the aflatoxin M1 peak. When acetonitrile–methanol–water (20:5:75, v/v/v), was used the two peaks could be separated efficiently, as is evident from Fig. 7. Detailed study showed that the peak at a retention time of 9 min is not due to aflatoxin B1, B2, G1, G2, M2, P1 or Q1, but is probably caused by the silicone glue used in the dialysis unit. The peak with the same retention time as aflatoxin M1 in the blank is probably due to an interferent dissolved from the dialysis membrane by milk constituents.

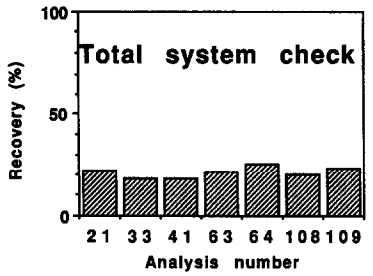

Fig. 8. Recovery as a function of the number of analyses performed with the same anti-aflatoxin immuno precolumn according to the hollow-fibre dialysis/immunoaffinity preconcentration procedure. Each data point corresponds to the analysis of a standard solution containing 300 ng/l of aflatoxin M1. For further explanation, see text.

The initial aim of installing a dialysis unit was to increase the lifetime of the immuno precolumn. As already indicated by the good reproducibility of the recoveries obtained with spiked milk samples, the immuno precolumn indeed showed good stability. For further confirmation, the performance of the immuno precolumn was checked regularly by analysing a standard solution containing 300 ng/l of aflatoxin M1 using the hollow-fibre dialysis/immunoaffinity preconcentration procedure. As this analyte amount was close to the total capacity of the immuno precolumn (*cf.*, Fig. 6), deactivation of the immuno precolumn should certainly lead to a decrease in recovery. Fig. 8 shows the recoveries found with the control experiments. No significant decrease in the capacity was observed during 109 runs performed with the same immuno precolumn. About 70% of all runs were used to analyse crude milk samples, and the remaining 30% for constructing calibration graphs and performing the control experiments. In other words, at least 70 milk analyses can be performed with a single immuno precolumn. Also, the hollow-fibre unit did not noticeably deteriorate owing to, *e.g.*, fouling or clogging of the membrane, during the stated number of analyses. This is a useful advantage of the present method over that of Tuinstra *et al.* [9], who had to use defatted milk in their on-line dialysis/C_{18} preconcentration system.

CONCLUSIONS

A flat membrane dialysis unit (flowing donor–flowing acceptor dialysis) and laboratory-made hollow-fibre dialysis unit (stagnant donor–flowing acceptor dialysis) were both coupled on-line to an immunoaffinity sample pretreatment LC system. With both systems overall recoveries of 6% were achieved for aflatoxin M1 in milk (dialysis time 220 min). Because the sample volume was about double with the hollow-fibre system, the detection limit was correspondingly lower. In addition, with milk samples the hollow-fibre system showed a better reproducibility of 3% R.S.D. compared with 12% for the flat membrane system. Both systems allowed the repeated analysis of milk samples without exchanging the immuno precolumn. With the hollow-fibre system, over 70 milk samples were analysed without observing any decrease in performance. The capacity of the immuno precolumn and the dialysis efficiency of the hollow-fibre unit remained constant during these runs, and no fouling or blocking of the dialysis membrane was observed.

The results demonstrate the applicability of the hollow-fibre system for the trace-level determination of aflatoxin M1 (detection limit 10 ng/l) in untreated milk. For unattended analysis, full automation of the system can be achieved by using an inexpensive peristaltic pump to flush the hollow-fibre dialysis unit.

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