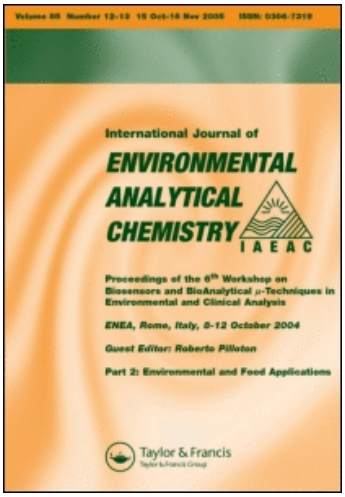

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IMMUNO PRECOLUMNS FOR SELECTIVE ON-LINE SAMPLE PRETREATMENT OF AFLATOXINS IN MILK PRIOR TO COLUMN LIQUID CHROMATOGRAPHY

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A column liquid chromatographic system for on-line sample pretreatment and determination of aflatoxin M1 in milk is described. The system consists of an immuno precolumn packed with immobilized monoclonal or polyclonal antibodies, a second precolumn packed with C-18 bonded silica and a C-18 analytical column. Defatted milk is directly loaded on the immuno precolumn. Desorption takes place by eluting the immuno precolumn with methanol-water (70:30, v/v). The eluate is diluted on-line with water with subsequent reconcentration of the analytes on the C-18 bonded silica precolumn and separation on the analytical column followed by fluorescence detection.

The applicability of automated immunoaffinity sample preparation with both a polyclonal and a monoclonal immuno precolumn is discussed and aspects such as the interference of milk proteins and the use of an external standard are investigated. Both types of immuno precolumns can be used for at least 20 analyses of milk samples, with relative standard deviations of 5–10% and detection limits of 20 ng/l for aflatoxin M1 in milk. The linear dynamic range is 20–400 ng/l of aflatoxin M1 in milk if 2.4-ml milk samples are used.

KEY WORDS: Immunoaffinity precolumns, column switching, column liquid chromatography, aflatoxins, milk

INTRODUCTION

Aflatoxins are highly toxic compounds which can cause cancer. The aflatoxins B1, B2, G1 and G2 are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi grow, for example, on peanuts, maize and leguminous plants; as a result aflatoxins can be present in animal and human foodstuffs[1]. Aflatoxin M1 is a hydroxylated metabolite of aflatoxin B1 and occurs in milk from cows which have been fed fodder contaminated with B1[1,2]. Aflatoxin M1 has an acute toxicity comparable to that of aflatoxin B1 and is a potential carcinogen[3]. Its presence in milk is therefore a hazard to human health. In the European Community the maximum allowable concentration of aflatoxin B1 in human foodstuff is 5 μg/kg[4]. In Austria and Germany the maximum allowable concentration for aflatoxin M1 in milk used for infants is 0.1 μg/kg.

The most commonly used method for aflatoxin analysis is column liquid chromatography (LC) with fluorescence detection[5]. The fluorescence sensitivity of the aflatoxins

B2, G2 and M1 is significantly higher than that of the aflatoxins B1 and G1. However, for the latter solutes a 20-fold increase in fluorescence sensitivity is observed after reaction with iodine or bromine[6,7]. This can be achieved in a post-column reaction detection system using on-line generated iodine[6] or bromine[7].

Normally, off-line liquid-liquid extractions and solid-phase isolations are used as sample pretreatment procedures, but these methods are rather laborious and difficult to automate[8,9]. Recently, antibodies against aflatoxins have become available for selective sample pretreatment. Mortimer *et al.*[10] described a procedure for the determination of aflatoxin M1 in milk using an immuno column containing an immobilized monoclonal antibody, for clean-up of the sample. Advantages of the method are high selectivity, short analysis time, low detection limit and high recovery. Recently it has been shown that precolumn techniques using immobilized antibodies can be automated[11,12]. Commercially available anti-aflatoxin immuno precolumns have also been used in such an on-line system[13]. Although these precolumns lose part of their capacity during the first few analyses, stabilization of the capacity is achieved after several runs, such that repeated and reproducible analysis of standard samples is possible. Unfortunately, in contrast to standard samples, the repeatability with milk samples is very bad.

In this paper the deteriorating effect of the milk matrix on immuno precolumns is investigated. A monoclonal and a polyclonal immunosorbent are characterized and compared for use in automated milk analysis. Different strategies are described for the reliable and repeated use of the immuno precolumns for the determination of aflatoxin M1 in milk.

EXPERIMENTAL

Apparatus

The set-up of the LC system is schematically shown in Figure 1. It consisted of two Gilson (Villiers-le-Bel, France) Model 302 pumps—one for the analytical column (pump 3) and one for the water dilution step (pump 2), an Applied Biosystems (Ramsey, NJ, U.S.A.) Spectroflow 400 pump (pump 1), a Spark Holland (Emmen, The Netherlands) PROSPEKT 1.0 programmer containing the valve switching units and the solvent selection valve, a Perkin–Elmer (Beaconsfield, U.K.) LS-4 fluorescence detector (λ_{ex} = 360 nm, λ_{em} = 440 nm; slit widths, 10 nm) and a Kipp & Zonen (Delft, The Netherlands) BD 40 recorder. In some cases a Perkin-Elmer Model 3000 fluorescence detector was used instead of the LS-2. The analytical column was a home-packed 100 mm × 3.0 mm I.D. glass column filled with 5 μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.), 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. C-18 stainless-steel precolumn was packed with 40 μm Baker (Deventer, The Netherlands) C-18 Bonded Phase material.

Separation was performed at ambient temperature using acetonitrile-methanol-water (16:24:60, v/v) as the mobile phase. The flow rate was 0.5 ml/min. The immuno

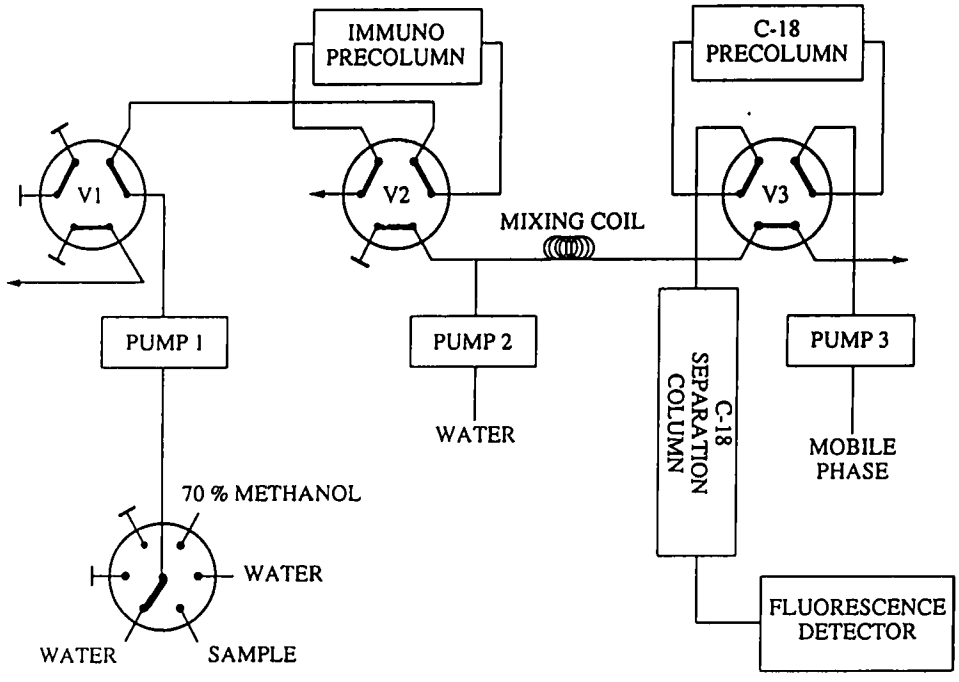

Figure 1 Schematic of automated immunoaffinity column switching system for the determination of aflatoxins.

precolumns were home-made 10 mm × 10 mm I.D. or 10 mm × 4.0 mm I.D. stainless-steel columns equipped with 5 μm stainless-steel screens and PTFE rings as the column inlet and outlet. The packing procedure of the immuno precolumns has been described elsewhere[11].

Chemicals

Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). Hexamethyltrimethylammonium chloride (cetrimide) was from Kodak (Rochester, NY, U.S.A.) and sodium azide was obtained from Merck. The aflatoxins were a gift from RIKILT (Wageningen, The Netherlands). Because of the high price of aflatoxin M1, optimization was performed with aflatoxin B2 where possible. LC-grade acetonitrile and methanol were purchased from Baker, LC-grade water was prepared from demineralized water using a Milli-Q (Millipore, Bedford, MA, U.S.A.) water purification system with subsequent filtration over a 500 mm × 10 mm I.D. LC column filled with 40 μm Baker C-18 Bonded Phase material. The LC eluents were degassed under vacuum in an ultrasonic bath. The monoclonal anti-aflatoxin antibodies were obtained in immobilized form from Koopman & Koek (Rijswijk, The Netherlands). The polyclonal sheep anti-aflatoxin antibodies were obtained from HyCult (Uden, The Netherlands).

The phosphate-buffered saline (PBS) stock solution contained 80 g of sodium chloride, 2 g of potassium chloride, 11.5 g of disodium hydrogen phosphate, 2 g of potassium dihydrogen phosphate and 20 ml of a 0.1 M sodium ethylenediaminetetraacetic acid solution per liter. The pH was adjusted to 7.4 with a 1 M sodium hydroxide solution.

Antiserum purification and immobilization of the polyclonal antibodies

Isolation of the antibody immunoglobulin G (IgG) fraction from the polyclonal sheep antiserum was based on the method of McKinney and Parkinson[14] with some minor modifications, as described by Farjam *et al.*[12] The recovery of IgG was 20.0 mg per ml antiserum and was determined by UV measurements. The purified IgG solution was stored in a 100-fold diluted PBS stock solution at −20°C.

The isolated IgG fraction was immobilized on cyanogen bromide-activated Sepharose 4B as recommended by the manufacturer[15]. 10 mg IgG per ml of wet gel were used for the immobilization. The immunosorbents were stored in a 0.1 M sodium phosphate buffer (pH 7.2) containing 0.02% (w/v) of sodium azide at 4°C.

Milk samples

Milk samples were purchased from grocer's shops. Before use, all milk samples were defatted by centrifugation at 3000 g during 15 min.

For precipitation of the milk proteins, the milk sample was acidified to pH 4.6 with 6 M hydrochloric acid. Subsequently the sample was heated for 15 min at 50°C and centrifuged at 160,000 g during 15 min. An aliquot of the yellow fluid between the upper fat layer and the lower protein layer was filtered through a 0.45 μm Milli Q filter (Millipore), after which a clear yellow fluid was obtained.

Analytical procedure

The analytical procedure for the on-line immunoaffinity sample pretreatment is described in detail in Ref. 13. In short, the analytical procedure when using, e.g. the 10 mm × 10 mm I.D. immuno precolumns is as follows (Figure 1). The first step involves preconditioning of the immuno precolumn with water. With the monoclonal immuno precolumn this step is performed with 5.0 ml of water during 1 min and with an additional 5.2 ml during the following 20 min, and with the polyclonal column with 10.2 ml during 5 min. Then the sample is introduced via pump 1 and the solvent selection valve. Next the immuno precolumn is flushed with 5.2 ml of water (pump 1) during 2 min to displace the remaining sample and to remove non-specifically bound impurities. Simultaneously the C-18 precolumn is preconditioned with 4.7 ml of water during 1 min via pump 2. Subsequently the immuno precolumn which now contains the trapped analytes, and the C-18 precolumn are switched in series and the immuno precolumn is desorbed with 2.1 ml of methanol-water (70:30, v/v) in the backflush mode using pump 1 during 8 min. During this process the methanol-water eluate is diluted with water to a methanol content of 3.7% by pump 2 in order to

allow reconcentration of the aflatoxins on the C-18 precolumn. After the quantitative transfer of the analytes to the C-18 precolumn, the actual separation is started by switching this precolumn on-line with the analytical column. Finally the immuno precolumn is flushed with 650 μl of methanol-water (70:30, v/v). The next analysis can be started while the separation in the analytical column is still running. The total time of analysis is 30 min when a polyclonal immuno precolumn is used, and 45 min with a monoclonal immuno precolumn.

RESULTS AND DISCUSSION

Monoclonal immuno precolumns

In a previous study[13] it was found that the commercial monoclonal immuno precolumns lost a significant part of their capacity (at least 90%) during the first few analyses. However, after five to six runs the capacity stabilized at a constant value For a 10 mm × 4.0 mm I.D. column, this value was about 10 ng for aflatoxin B. With such a 'stabilized' immuno precolumn repeated analysis of standard samples is possible. Table 1 shows the analytical data which were obtained for the analysis of 10 ml of standard samples with a stabilized 10 mm × 10 mm I.D. monoclonal immuno precolumn. The recovery values of about 80% for both analytes are rather satisfactory and the same is true for the repeatability of 2.5–3%. These values did not noticeably change even when performing 50 runs with the same immuno precolumn.

If spiked milk samples were analyzed under similar conditions, a constantly decreasing capacity was observed. Figure 2 shows the recovery as a function of the number of analyses and the type of sample analyzed (aqueous standard or milk). After stabilization of the immuno precolumn with aqueous samples (analyses Nos. 1 to 9), the next three analyses (Nos. 10–12) performed with 8 ml of standard sample containing 1.25 μg/l of aflatoxin M1 showed a constant recovery of 77 $\pm$ 1%. With the first milk sample (No. 13), the recovery started to drop. This was found for both the milk samples and the standard samples analyzed in between the milk runs. The aflatoxin M1 recovery dropped from 65% with the first milk analysis to 42% with the ninth milk analysis. The aflatoxin M1 recovery with standard samples dropped

Table 1 Analytical data for the on-line immunoaffinity sample pretreatment with 10 ml aqueous samples using a 10 mm × 10 mm I.D. monoclonal immuno precolumn.

Criterion	*Aflatoxin M1*	*Aflatoxin B2*
Linear dynamic range	0.02–10 ng	0.002–30 ng
Recovery	77%	88%
Repeatability (RSD)	3%; $n = 6$ (level, 5 ng)	2.5%; $n = 6$ (level, 10 ng)

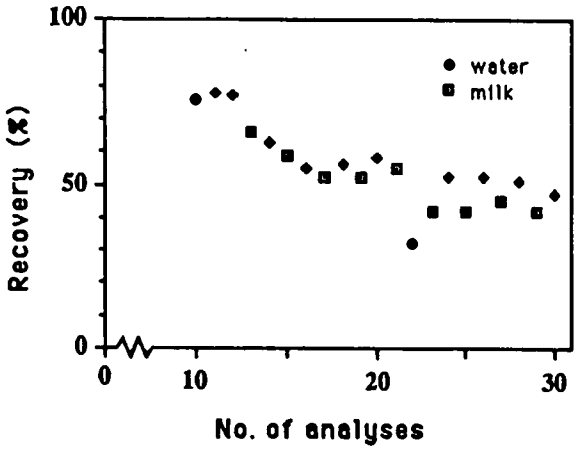

Figure 2 Recovery as a function of the number of analyses and type of sample. The on-line immunoaffinity sample pretreatment was performed with a 10 mm × 10 mm I.D. monoclonal immuno precolumn 8 ml of sample spiked with 1.25 μg/l of aflatoxin M1 were analyzed. The first nine analyses (not shown) were performed with aqueous samples.

from the initial 77% to 47% after the ninth milk analysis. Since the recovery decrease was also found for the aqueous standard samples analyzed in between the milk runs, one must conclude that the immuno precolumn is slowly deactivated by contact with the milk samples. The RSD for the nine milk analyses in Figure 2 was poor, i.e. 18%. Similar results were obtained if milk samples spiked with aflatoxin B2 were analyzed.

If the results of the analyses of the aflatoxin M1-spiked water samples preceding and following a milk analysis were chosen as external standards, the RSD for the milk sample analyses significantly improved to 6.5% ($n = 9$ for milk samples). A drawback of such a procedure, of course, is that the time of analysis of a milk sample increases two-fold.

In order to investigate if the decrease of the immuno column capacity is due to milk proteins, acid-precipitated milk samples were analyzed. The results are shown in Table 2. If a milk sample was spiked with 1 μg/l of aflatoxin B2 after precipitation,

Table 2 Analytical data for aflatoxin B2 in acid-precipitated milk samples using a 10 mm × 10 mm I.D. monoclonal immuno precolumn.

Conditions	*Recovery (%)*	*RSD (%)*
Milk spiked after precipitation repeated analysis of the same batch	85	6 ($n = 7$)
Milk spiked before precipitation; repeated analysis of the same batch	19	7 ($n = 5$)
Milk spiked before precipitation; same sample analyzed but separate precipitation	26	22 ($n = 4$)

no decrease in recovery was found if the sample was analyzed repeatedly. A recovery of 85% and a RSD of 6% ($n = 7$) were found. If the milk sample was spiked before precipitation and the resulting supernatant was repeatedly analyzed, a similar repeatability was achieved, but the recovery dropped dramatically to about 20%. Obviously the aflatoxin was lost during precipitation due to occlusion and/or coprecipitation. In addition, testing the batch-to-batch reproducibility in a series of experiments in which each spiked milk sample was subjected to acid precipitation separately, gave a disappointingly high RSD of 22% ($n = 4$). Since the procedure was, also, rather time consuming (cf. Experimental section), it was not further investigated.

In another set of experiments, 2 mM (twice the CMC) of the cationic surfactant hexadecyltrimethylammonium chloride was added to spiked milk samples before loading them on the immuno precolumn to test whether the micellar solution can solubilize the interfering milk components. Unfortunately the aflatoxin recovery dropped to zero if the surfactant was present in a sample. Loading and analyzing micelle-free samples after these experiments, showed that the immuno precolumn capacity had not been irreversibly destroyed by the micellar solution: the capacity was completely restored if micelle-free solutions were preconcentrated.

Polyclonal immuno precolumns

It is well known that the properties of antibodies can vary significantly from one strain to another. Therefore the behaviour of a commercially available polyclonal anti-aflatoxin antibody was also studied.

Initial experiments showed that, in marked contrast with the monoclonal immuno precolumn, the polyclonal precolumn did not undergo a capacity drop during the first runs. To quote one example, the capacity of a freshly packed 10 mm × 10 mm I.D. polyclonal immuno precolumn remained constant if a 10 ml standard sample containing (a large excess of) 500 ng of aflatoxin B2 was repeatedly analyzed (RSD = 4.7%, $n = 4$).

Another significant difference was found with respect to the water flushing step, which was performed to displace the methanol used for desorption and to regenerate the activity of the immuno precolumn before the next analysis. While with the monoclonal immuno precolumn the efficiency of regeneration was almost linearly dependent on the water flush step[13], this was not found with the polyclonal immuno precolumn. Performing this step for 2 to 20 min (5.5 ml of water used for each run) with the 10 mm × 4.0 mm I.D. immuno precolumn gave the same recovery of 47 ± 3% (0.5 ng of aflatoxin B2 loaded from 10 ml of standard sample) in all cases. Obviously, both the capacity loss during early runs and, somewhat more surprisingly, the regeneration behaviour of the immuno precolumns are characteristic parameters, which probably primarily depend on the type of antibodies used and the nature of the immobilization process.

The analytical data for the polyclonal immuno precolumn, as determined with spiked standard samples, are shown in Table 3. Compared with the monoclonal precolumn, the polyclonal precolumn, because of a lower capacity, has a distinctly

Table 3 Analytical data of the on-line immunoaffinity sample pretreatment with 10 ml aqueous samples using a 10 mm × 10 mm I.D. polyclonal immuno precolumn.

Criterion	*Aflatoxin M1*	*Aflatoxin B2*
Linear dynamic range	0.04–1.5 ng	0.002–0.5 ng
Recovery	46%	97%
Repeatability (RSD)	4%; $n = 6$ (level, 0.5 ng)	2.8%; $n = 6$ (level, 0.5 ng)

shorter linear dynamic range for both aflatoxin M1 (up to 1.5 ng) and aflatoxin B2 (up to 0.5 ng). As regards the recovery, for aflatoxin M1, it was significantly lower than with the monoclonal precolumn, while for aflatoxin B2 it was somewhat higher. Presumably, the explanation of the seemingly contradictory data is that the polyclonal precolumn has a relatively high affinity for aflatoxin B2, while the absolute number of available antibody-binding sites is small.

When analyzing milk samples, the polyclonal precolumn showed a better stability than the monoclonal precolumn, as becomes clear upon comparing Figures 2 and 3. From the latter figure, one can read a recovery decrease from 47% for the first milk analysis to 39% for the tenth. If 8 ml of milk spiked with 63 ng/l of aflatoxin M1 were analyzed, the RSD for the milk analyses was 10% ($n = 10$). If—as was also done with the monoclonal precolumn—the results for the spiked water samples preceding and following each milk analysis were taken as external standards, the RSD for the milk analyses improved to 4.4% ($n = 10$). Another possibility to improve repeatability was to use aflatoxin B2, which normally does not occur in milk, as an internal standard. If 2.4 ml of milk sample spiked with 200 ng/l of aflatoxin M1 and with an

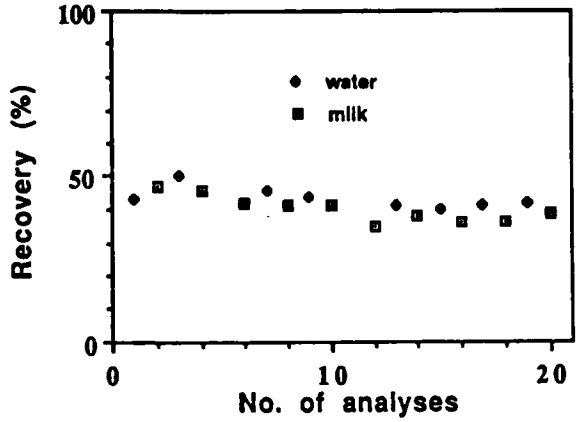

Figure 3 Recovery as a function of the number of analyses and type of sample. The on-line immunoaffinity sample pretreatment was performed with a 10 mm × 10 mm I.D. polyclonal immuno precolumn. 8 ml of sample spiked with 63 ng/l of aflatoxin M1 were analyzed.

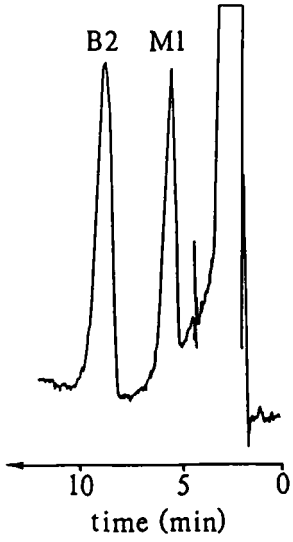

Figure 4 LC chromatogram of a 2.4-ml milk sample spiked with 200 ng/l of aflatoxin M1 and 20 ng/l of aflatoxin B2. A 10 mm × 10 mm I.D. polyclonal immuno precolumn was used for sample pretreatment. For the LC and fluorescence detection conditions, see the Experimental section.

additional 20 ng/l of aflatoxin B2 as an internal standard, were repeatedly analyzed, an RSD of 5.5% ($n = 20$) was found (Figure 4).

The above results show that direct loading of milk samples is possible with the polyclonal immuno precolumn. Acceptable RSD values can be obtained both with an external and an internal standard. At least 20 milk analyses could be performed on the same precolumn. The only real disadvantage of the present polyclonal precolumn is its limited linear dynamic range. With milk samples linearity was observed up to 1.0 ng of aflatoxin M1. Still, this implies that, if e.g. 2.4 ml of milk are preconcentrated, a linear dynamic range of between 20 ng/l and 400 ng/l can be achieved for aflatoxin M1, which will be adequate for most purposes.

CONCLUSIONS

In this paper, a monoclonal and a polyclonal anti-aflatoxin immuno precolumn have been compared for use in the on-line automated sample pretreatment/LC determination of aflatoxin M1 in milk. Apart from their widely divergent capacities—and, consequently, linear dynamic ranges—the two immuno precolumns showed other significant differences. With aqueous samples the monoclonal immuno precolumn suffered a capacity drop of about 90% during the first six to ten analyses; after that, plateau conditions had been reached and no further loss occurred during at least 50 runs. The polyclonal immuno precolumn, on the other hand, maintained its initial capacity without any real loss during a similar number of runs. Further, whereas the regeneration with water of the monoclonal immuno precolumn was a slow process

which was almost linearly dependent on the flush time, no such dependence was found with the polyclonal immuno precolumn.

With spiked milk samples both immuno precolumns showed a decreasing recovery upon repeated use, which must be attributed to interfering milk constituents. The loss of recovery was, however, much more significant with the monoclonal compared with the polyclonal immuno precolumn. The problem caused by the loss of recovery could partly be eliminated by running an aqueous sample after each milk sample, using these as external standards. Under these conditions, quite satisfactory RSD values of about 5% were obtained with both types of immuno precolumns, and at least 20 analyses of milk samples could be carried out automatedly on a single precolumn; this implies a considerable reduction of the running cost per analysis. As for detection sensitivity, with 2.4-ml milk samples, the present immuno precolumn/LC set-up allows the detection of 20 ng/l of aflatoxin M1 in milk.

Two further conclusions can be drawn from the present study. Firstly, one has to realize that antibodies are more or less natural products, with properties which strongly depend on the production batch. Therefore, every new antibody has to be evaluated with respect to the parameters which are primarily important for the application which is envisaged. With the present on-line immuno precolumn/LC system, the capacity of an antibody in immobilized form, its stability during methanol desorption as well as with respect to type of sample to be analyzed, and the ease of regeneration after desorption appear to be the most prominent aspects. Both the present and earlier[10–13, 16] studies demonstrate that, although 'ideal' antibodies are not easily obtained, antibodies which meet the various demands to some degree are available on the market. Secondly, it is obvious that the external standardization discussed above, causes an undue increase of the time of analysis of a milk sample. In a subsequent study, we shall therefore attempt to remove the interfering milk constituents—probably proteins—by means of a dialysis step coupled on-line with the present pretreatment/LC procedure.

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