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Determination of ochratoxin A in urine and faeces of swine by high-performance liquid chromatography

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ABSTRACT

Sensitive methods for the determination of ochratoxin A in urine and faeces of swine are described. The samples were extracted with chloroform at pH <2, and the extracts were cleaned up by a combination of solid-phase extraction and liquid–liquid partition. High-performance liquid chromatography with fluorescence detection was used for detection and determination. The detection limits were 0.3 ng/ml for urine and 1.5 ng/g for faeces. Recoveries of ochratoxin A from spiked samples of urine and faeces were 93% and 60%, respectively. Because of the low detection limit and the fast and relatively easy performance, the method for the determination of ochratoxin A in urine proved suitable for the estimation of possible contamination of live animals.

INTRODUCTION

Ochratoxin A, a secondary fungal metabolite produced by several species of *Aspergillus* and *Penicillium* during the storage of foods and feeds, is a potent nephrotoxin [1,2]. In addition, immunosuppressive, teratogenic and carcinogenic effects have been reported [3–5]. Swine are very sensitive to contamination with ochratoxin A; after intake of the toxin with contaminated feed, residues in blood, kidney, liver and muscle can be found [6,7]. Thus, exposure of humans takes place not only through contaminated plant products, but also indirectly through residues in slaughter animals. In some instances, ochratoxin A has been detected in human blood and breast milk [8].

Many methods have been described for the determination of ochratoxin A in blood serum and kidneys of swine (animal tissues with the highest residues of ochratoxin A), including thin-layer chromatography (TLC) [9–11], high-performance liquid chromatography (HPLC) [8,12–14], radioimmunoassay [15] and enzyme-linked immunosorbent assay (ELISA) [16].

Few studies have been reported which deal with the determination of ochratoxin A in urine or faeces of livestock. As a non-invasive procedure, this determination could give information about possible contamination of live animals. Satisfactory results, relating to the limit of detection, could not be obtained either by TLC [17–19] or by HPLC with a TLC clean-up procedure [20,21]. Orti *et al.* [22] and Castegnaro *et al.* [23] used very sensitive HPLC methods for the analysis of human urine, but they involved very complicated clean-up procedures.

The aim of this study was to develop HPLC methods for the determination of low ochratoxin A concentrations in urine and faeces. Emphasis was laid on the clean-up procedures, which should be rapid and as easy as possible. These methods have been used for the analysis of samples from a feeding experiment with growing pigs

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with low-contaminated feedstuff (100 μg/kg); the results of this experiment will be published elsewhere [24].

EXPERIMENTAL

Reagents

Ochratoxin A was obtained from Makor Chemicals (Jerusalem, Israel). A stock solution in methanol (100 μg/ml) was diluted with methanol to a concentration of 10 μg/ml and assayed spectrophotometrically ($\varepsilon_{333\ nm}$ = 6640 [8]). Ochratoxin A–antiserum and ochratoxin A–enzyme-conjugate for the ELISA method were obtained from the Institut für Hygiene und Technologie der Milch, Universität München (Munich, Germany). For enzymatic hydrolysis, β-glucuronidase, type H-1 (activity 300 000–400 000 U/g, sulphatase activity 15 000–40 000 U/g) from Sigma (Deisenhofen, Germany) was used; 50 mg were dissolved in 10 ml of 0.2 *M* acetate buffer (pH 5.5). All other chemicals and solvents were of analytical-reagent grade, except acetonitrile, which was of HPLC grade. Silica gel Si 400 cartridges were obtained from Merck (Darmstadt, Germany), and poly(vinylidene fluoride) (PVDF) membrane syringe filters (0.45 μm) for HPLC from Millipore (Eschborn, Germany) or Amchro (Sulzbach, Germany).

Urine and faeces samples

Contaminated samples were available from a feeding experiment with growing pigs. The animals were fed a diet containing ochratoxin A at a concentration of 100 μg/kg for 85 days. In several periods, urine and faeces were collected daily, and samples (urine after acidification) were kept in a deep freezer at −18°C until analysed. For recovery experiments, samples from animals fed a toxin-free diet were used. Faeces were lyophilized and ground before analysis.

Apparatus

For checking the titre of the ochratoxin A standard solution, a Model 554 UV–Vis spectrophotometer from Perkin-Elmer was used. It was calibrated according to an AOAC procedure [25].

The HPLC equipment was purchased from Kontron Instruments (Neufahrn, Germany) and consisted of a pump (Type 420), an autosampler (Type 360), a fluorescence spectrophotometer (SFM 25) as the detector and a data system (450-MT). A SuperPac Spherisorb ODS-2 Cartridge (250 mm x 4 mm I.D.; 5 μm), in combination with an ODS-2 guard column (10 mm x 4 mm I.D., 5 μm), both from Pharmacia Biosystems (Freiburg, Germany), was used as the analytical column.

For the ELISA determinations, Immunoplate II microtitre plates from NUNC (Wiesbaden, Germany), an MR 5000 microplate reader from Dynatec (Denkendorf, Germany) and an Ultrawash II microplate washer from Dynatec were employed.

Extraction and clean-up of urine samples

Extraction of urine samples was performed by a modified method of Bauer and Gareis [8] for blood serum. A 20-ml portion of urine was mixed with 20 ml of 0.4 *M* magnesium chloride solution and acidified to pH 1.6–2.0 with 2 *M* hydrochloric acid. After addition of 20 ml of chloroform, the flask was shaken automatically for 30 min and then clarified by centrifugation at 1700 *g* for 25 min.

For the clean-up, a combined procedure of solid-phase extraction and liquid–liquid partitioning was used. A 15-ml aliquot of the chloroform extract was run through a silica gel cartridge, prewetted with chloroform. The column was washed with 10 ml of chloroform, and ochratoxin A was subsequently eluted with 10 ml of methanol–20 % formic acid (9:1, v/v). The eluate was evaporated nearly to dryness on a rotary evaporator and the residue dissolved in 6 ml of 0.1 *M* sodium hydrogencarbonate solution. After acidification with concentrated formic acid to pH 1.6–2.0, the solution was extracted twice with 2 ml of chloroform; for clarification, the tube was centrifuged at 1200 *g* for 5 min. The combined chloroform layers were evaporated to dryness on a rotary evaporator and the residue was dissolved in 0.5 ml of methanol and filtered through a membrane filter.

Enzymatic hydrolysis of urine for the detection of conjugated ochratoxin A

This was performed according to a modification of the procedure of Orti *et al.* [22]. A 10-ml volume of urine was mixed with 3 ml of 0.2 *M* sodium sulphate solution and 4 ml of 0.2 *M* acetate buffer (pH 5.5) and the pH was checked. Then 3 ml of β-glucuronidase solution were added and the mixture was incubated overnight at 37°C. The hydrolysed urine was extracted according to the procedure above, but without addition of magnesium chloride solution, and the extract was cleaned up by the combined procedure described above.

Extraction and clean-up of faeces samples

A 4-g sample of lyophilized and ground faeces was extracted according to the procedure described for urine, except that 40 ml of chloroform were used. A combination of liquid–liquid partition by a modified method of Bauer and Gareis [8] and solid-phase extraction [26] was applied for the clean-up, as follows.

A 15-ml aliquot of the chloroform extract was extracted twice with 10 ml of 0.1 *M* sodium hydrogencarbonate solution; for clarification, the tube was centrifuged at 1200 *g* for 10 min. The combined aqueous layers were acidified with concentrated formic acid to pH 1.6–2.0 and re-extracted twice with 5 ml of chloroform. The combined chloroform extracts were dried over 1 g of sodium sulphate and evaporated to dryness on a rotary evaporator.

The residue was taken up in 3 ml of toluene and run through a silica gel cartridge, prewetted with toluene. The column was washed with 5 ml of toluene and 12 ml of toluene–acetone (95:5, v/v). Ochratoxin A was subsequently eluted with 12 ml of toluene–acetone–formic acid (90:10:5, v/v/v) and the eluate was evaporated to dryness on a rotary evaporator. The residue was dissolved in 1 ml of methanol and filtered through a membrane filter.

HPLC analysis

HPLC with fluorescence detection was used for the determination of ochratoxin A. A 20-μl volume of the extract was injected into the chromatograph. Acetonitrile–isopropanol–0.083 *M* orthophosphoric acid (47:10:43, v/v/v) (modification of the eluent described by Lepom [27]) was used as the mobile phase for urine samples. For the analysis of faeces samples, acetonitrile–0.008 *M* orthophosphoric acid (56:44, v/v), adjusted to pH 2.8 with orthophosphoric acid, was used. The flow-rate was 1 ml/min in both instances. The excitation wavelength of the fluorescence detector was set at 330 nm and the emission wavelength was 460 nm.

The concentration of ochratoxin A was calculated by the data system by means of a linear multi-level calibration graph in the range 1–100 ng/ml, based on peak area.

Confirmation by ELISA

For confirmation of ochratoxin A, a defined volume of the purified sample extract in methanol was evaporated to dryness and the residue was dissolved in a volume of 0.13 *M* sodium hydrogencarbonate solution such that the resulting concentration of ochratoxin A was in the range 0.05–1.00 ng/ml. The determination by ELISA was performed according to the method of Märtlbauer [16] for blood serum and kidneys. For each sample, eight wells of a microtitre plate were used.

RESULTS AND DISCUSSION

Method for determination of ochratoxin A in urine

The chloroform extraction at pH < 2, which is widely used for serum and kidneys [8], proved to be suitable for urine also. The addition of magnesium chloride reduced the extraction of matrix interfering substances.

The clean-up by liquid–liquid partitioning [8], which was successful for a number of animal tissues [28], proved to be inadequate for eliminating interfering substances in urine. Solid-phase extraction on a silica gel cartridge with the elution mixture toluene–acetic acid (9:1, v/v), as described for cereals and blood serum [11,29], resulted in low recoveries and poor reproducibility.

The combined method of solid-phase extrac-

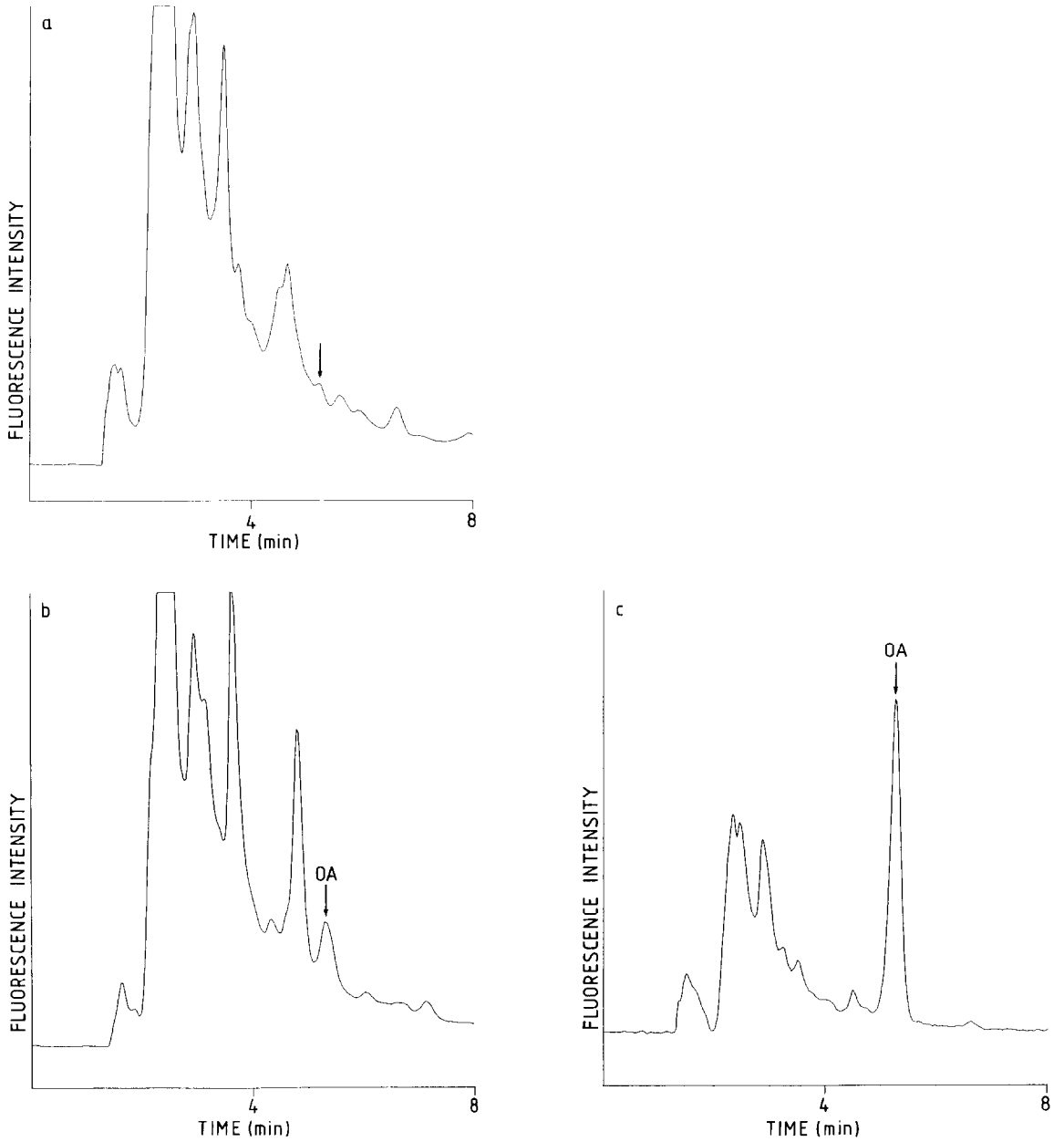

Fig. 1. HPLC of (a) a blank urine sample, (b) a urine sample spiked to contain 1 ng/ml ochratoxin A (OA) and (c) a urine sample naturally contaminated with 16 ng/ml ochratoxin A (1:3 diluted). Column, SuperPac Spherisorb ODS-2, 5 μm (250 mm × 4 mm I.D.); mobile phase, acetonitrile–2-propanol–0.083 *M* orthophosphoric acid (47:10:43, v/v/v); detector, spectrofluorimeter, excitation at 330 nm, emission at 460 nm.

tion on a silica gel cartridge with the elution mixture methanol–20% formic acid (9:1, v/v), followed by a single liquid–liquid extraction step, yielded the best results with regard to recovery and reproducibility, and effected a sufficient elimination of interfering substances. In addition, it was not necessary to dry the eluate completely, because the eluting mixture was miscible with the sodium hydrogencarbonate solution in which the residue was to be dissolved.

The HPLC eluent acetonitrile–2-propanol–0.083 *M* orthophosphoric acid (47:43:10, v/v/v) was chosen from a series of tested mobile phase mixtures (acetonitrile–dilute orthophosphoric acid with addition of 2-propanol, methanol or tetrahydrofuran), because with this eluent the best separation and quantification of the ochratoxin A peak could be achieved in most instances. In a few instances, the eluent used in the analysis of faeces yielded better results.

In Fig. 1, chromatograms of (a) a blank urine sample, (b) a sample spiked to contain 1 ng/ml ochratoxin A and (c) a naturally contaminated urine sample with a concentration of 16 ng/ml are presented. As shown in Fig. 1b, even small amounts of ochratoxin A can be determined satisfactorily, although the peak appears on the tail of a matrix peak. For higher concentrations (Fig. 1c), no interference with background peaks occurs.

At a signal-to-noise ratio of 3:1, the detection limit of the method was 0.3 ng/ml. For recovery estimates, urine samples from animals fed ochratoxin-free diets were supplemented, prior to the extraction, with suitable amounts of a standard solution of the toxin in the range 1–20 ng/ml in urine. The average recovery was 93%. The results are detailed in Table I.

The possible conjugation of ochratoxin A with glucuronic acid in urine, observed with other mycotoxins, *e.g.*, aflatoxins and zearalenone [30,31], was investigated. Two naturally contaminated samples were hydrolysed with β-glucuronidase before the extraction. The measured value of ochratoxin A in these samples was not increased by enzymatic pretreatment, suggesting the absence of such a conjugation mechanism *in vivo*.

TABLE I

RECOVERY OF OCHRATOXIN A FROM URINE

Urine samples of pigs fed an ochratoxin-free diet before the extraction were spiked with an ochratoxin A standard solution.

Concentration range of the sample (ng/ml)	Number of samples	Mean recovery (%)
1–3	14	85 ± 7
4–7	9	100 ± 4
10–20	9	99 ± 10

For confirmation of ochratoxin A in urine samples, an ELISA method developed by Märtlbauer [16] for the analysis of blood serum and kidneys was adopted. Although other techniques for confirmation, such as methylation of ochratoxin A to ochratoxin A methyl ester followed by identification of this derivative by HPLC [8,32], or scanning the fluorescence spectrum [33], have been described, only the ELISA method is suitable for confirming positive ochratoxin A results in the low-concentration range. The conformity between the results of the two methods was very good; for 33 positive urine samples in the range 0.3–26 ng/ml, the regression equation was: $y = -0.05 + 1.19x$ ($r^2 = 0.97$), y = concentration measured by ELISA and x = concentration measured by HPLC.

The method described allows very low concentrations of ochratoxin A in urine of swine to be determined. In addition, the method is rapid and relatively easy to perform and the recovery is very good. As even diets low in ochratoxin A (100 μg/kg) resulted in concentrations of 14–26 ng/ml ochratoxin A in the urine of pigs [24], this method appears suitable for detecting a possible ochratoxin A load in live animals.

Method for determination of ochratoxin A in faeces

With faeces, as for urine, the extraction with chloroform at pH < 2 proved to be suitable, but for the clean-up a more effective method was necessary. Fig. 2a shows a chromatogram of a faeces sample spiked with 4 ng/g ochratoxin A after a clean up by liquid–liquid partitioning according to Bauer and Gareis [8]; interpretation of the chromatogram is not possible because of the interfering matrix peaks. The clean-up procedure developed for urine samples was also inadequate, because the elution mixture for ochratoxin A on the silica gel cartridge (methanol–20% formic acid) was too polar and interfering polar matrix substances were also eluted.

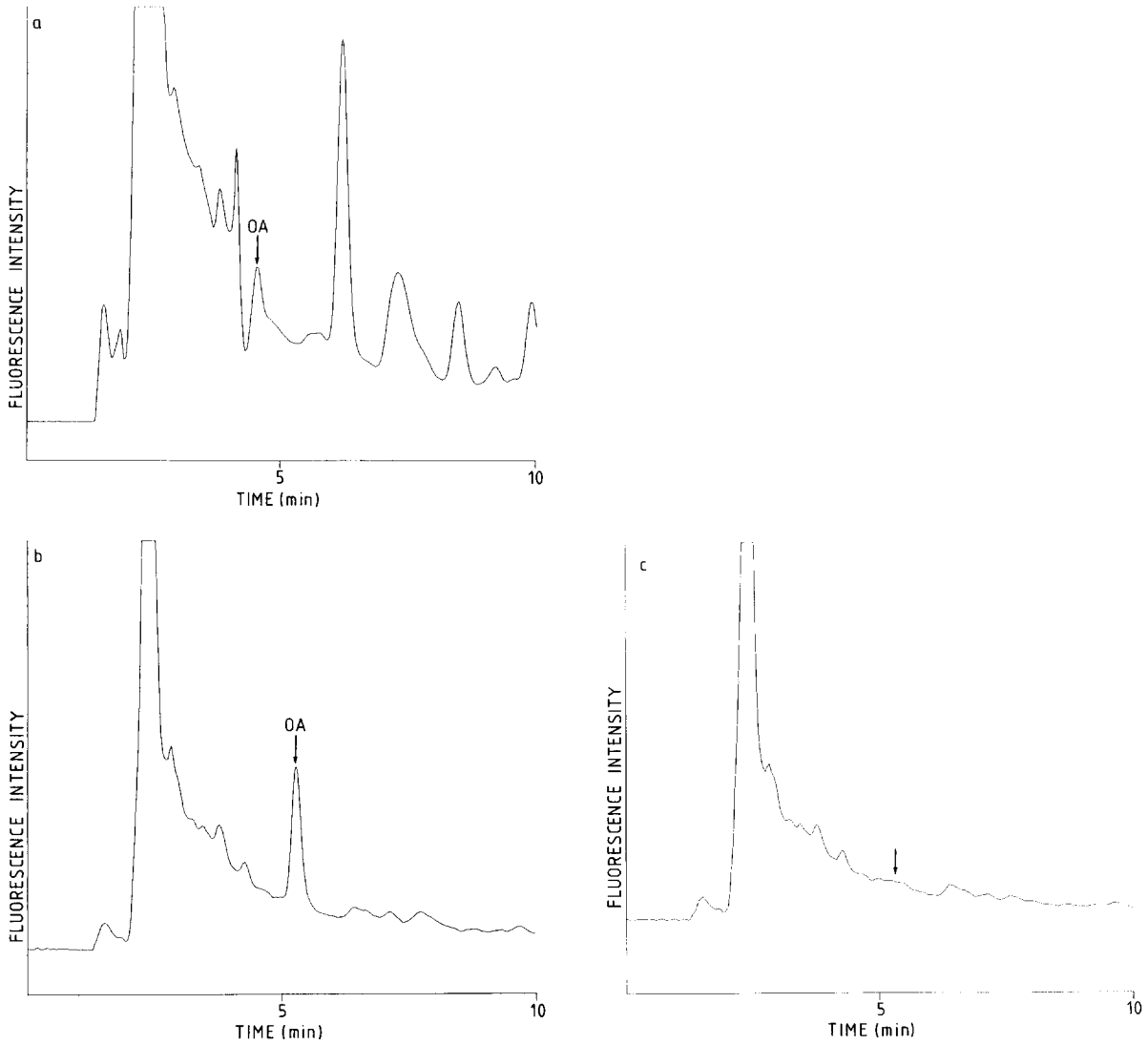

Fig. 2. HPLC of (a) a faeces sample spiked to contain 4 ng/g ochratoxin A (OA) related to fresh faeces, clean-up by liquid liquid partitioning according to Bauer and Gareis [8], (b) a faeces sample spiked to contain 8 ng/g ochratoxin A, clean-up by liquid–liquid partitioning and solid-phase extraction, and (c) a blank faeces sample, clean-up as for (b). Chromatographic conditions as in Fig. 1, except that acetonitrile–water–acetic acid (57:41:2, v/v/v) was used as mobile phase in (a) and acetonitrile–0.008 *M* orthophosphoric acid (56:44, v/v), adjusted to pH 2.8 with orthophosphoric acid, in (b) and (c).

The best results were achieved with a combined clean-up procedure consisting of a liquid–liquid-partitioning and a solid-phase extraction in the reverse order of that for urine samples. The solid-phase extraction had to be performed after the liquid–liquid step in order not to overload the silica gel cartridge with matrix components. The clean-up procedure by solid-phase extraction was based on a method [26] for cereals and feeds.

Experiments with spiked samples of faeces showed that ochratoxin A from the chloroform extract adsorbed completely on the silica gel, and that the elution with toluene–acetone–formic acid was nearly complete. However, we observed

TABLE II

RECOVERY OF OCHRATOXIN A FROM FAECES

Samples of lyophilized and ground faeces of pigs fed an ochratoxin-free diet before the extraction were spiked with an ochratoxin A standard solution.

Concentration of the sample[a] (ng/g)	Number of samples	Mean recovery (%)
2	10	54 ± 7
4	6	61 ± 6
6	8	64 ± 7

[a] Related to fresh faeces.

that adsorption on silica gel was complete only if the chloroform extract was evaporated completely to dryness after the liquid–liquid partitioning, before the residue was taken up in toluene. Otherwise, the recovery and reproducibility were very poor.

When mixtures of toluene with more polar solvents than acetone (methanol, ethyl acetate) were used for washing the column, more matrix substances were eliminated, but occasionally irreproducible loss of the toxin was observed. Langseth *et al.* [29] reported similar results with chloroform–methanol (97:3, v/v) in a method for the simultaneous determination of aflatoxins, ochratoxin A and zearalenone. Therefore, the original toluene–acetone mixture was retained.

Fig. 2b and c show chromatograms of a sample of faeces spiked with 8 ng/g ochratoxin A and of a blank faeces sample, respectively, after clean-up according to the procedure described above. It illustrates the clean-up effect of the solid-phase extraction; compared with the chromatogram in Fig. 2a, the interfering matrix peaks are largely eliminated.

The detection limit of the method was 1.5 ng/g, related to fresh faeces (signal-to-noise ratio = 3:1). The recovery is shown in Table II. The lower average recovery of 60% in comparison with urine was probably caused by the more complex clean-up procedure and the more difficult matrix. In the positive samples of faeces ochratoxin A was confirmed by the ELISA method, which was described above. Because only a few positive faeces samples were available, no correlation between the HPLC and ELISA results could be established.

The method described above allows, for the first time, ochratoxin A to be detected in faeces at concentrations as low as 1.5 ng/g. Thus, contamination caused by ochratoxin A can be reliably detected in faeces of pigs at this level, *e.g.*, in pharmacokinetic studies. However, the concentrations of ochratoxin A in faeces of pigs fed a diet containing as little as 100 μg/kg ochratoxin A were below this level in most instances [24]. Therefore, the measurement of ochratoxin A in the urine of pigs by the method described above is more appropriate for the determination of possible contamination of the animals.

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