Liquid Chromatographic Determination of Ochratoxin A

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Summary

Ochratoxin A is a potential carcinogenic mycotoxin. When grain contaminated with ochratoxin-producing mould species is used for pig feed, ochratoxin residues may be present in pork for human consumption. The present work describes an analytical procedure for quantifying ochratoxin in pork kidney, muscle and blood.

A standard curve for each matrix is obtained by spiking uncontaminated matrices with different concentrations of ochratoxin A dissolved in distilled water. The equilibration time is 15 minutes. Blood is spiked as whole blood and plasma is collected after centrifugation. To extract ochratoxin from plasma celite is added and samples are acidified with hydrochloric acid. The samples are extracted with 1:2 hexane: tert. butyl acetate. Kidney and muscle samples are acidified with phosphoric acid and sonificated to disperse the tissue. For tissue samples celite is added to bind proteins and major cell components. The ochratoxin is extracted with 1:1 hexane: ethyl acetate. The ochratoxin is then extracted from the organic phase with aqueous sodium bicarbonate and after acidification ochratoxin is back-extracted to an organic phase. (Plasma samples: Chloroform; tissue samples: tert. butyl acetate). The organic phase is evaporated and the residue is dissolved in the eluent (48:52 acetonitrile: acetic acid). Samples are injected to a HPLC-system (C-18 column) and detected by fluorescence (Em. 460 nm, Ex. 330 nm). Unfortunately, unspiked samples show a small peak at the same retention time as ochratoxin A. The samples have been run in four different eluent systems. The peak from the unspiked sample persists retending at the same time as does the ochratoxin A.

The samples have to be analyzed by MS before any conclusion on the small peak can be drawn. The background is subtracted recovery before calculations. The method gives a linear response for blood samples between 1 and 50 ppb and for tissue samples between 5 and 40 ppb. Correlation coefficients are always better than 0.990. Recovery is between 60 and 90%, being rather constant within a day.

Introduction

The Danish meat industry has recognised the increasing demands from governments and consumers for residue free food. To meet these demands and to use them as a competition parameter the industry has decided to develop a system, which is able to check as many carcasses as possible for unwanted residues. Such a system must be sufficiently fast to follow the slaughter speed and in addition be sturdy and give the best possible identification of the residues. Mass spectrometry (MS) is the only technique that meets these demands.

The system will be based on blood samples as it is easier to analyze blood than tissue. During the development of the MS-procedures it is necessary to use "reference" methods to ensure that no systematic error occurs. In addition it is necessary to study the ratio between concentrations in blood and tissue as most regulatory limits are set for tissue.

The present work describes a "reference" method for ochratoxin A. Ochratoxin A is a potential carcinogenic mycotoxin, produced under certain conditions by mould species that may be present on grain. When such grain is used for pig feed, ochratoxin residues may be present in pork.

If a pig has been exposed to ochratoxin for some time, the macroscopic structure of the kidney will change. The kidney becomes pale and deformed.

Today, Denmark is one of the few countries in the world routinely analyzing for ochratoxin residues in pigs. All animals with abnormal kidneys are analyzed. If a kidney contains more than 25 ppb, the whole carcass is condemned. The analytical method is a TLC-fluorescence procedure with liquid-liquid extraction for clean-up (1). Unfortunately, this procedure is quite time-consuming, and it does not give a 100% identification of the compound. A secondary problem is that the clean-up for this procedure, like other published methods (2-5) for animal matrices requires sizeable amounts of chloroform. Chloroform is potential carcinogen.

Only one larger study has been found in the literature (6) on the ratio between concentrations in blood and muscle and in blood and kidney. According to Mortensen et al. (6) the ratio between serum and kidney is between 9 and 15, increasing for increasing serum levels. Between serum and muscle the ratio is 13-34 with a bias towards 30, but no clear trends as far as serum level is concerned. The variations described might be due to natural variations or might be caused by a less sturdy analytical procedure.

Purpose

The purpose of this study is to develop an easier clean-up without the use of chloroform and to develop a HPLC procedure for quantification of the detected compound. Methods are developed for muscle, kidney and blood in order to check the ratios found by Mortensen et al. later on. For abnormal kidneys the results are compared to the TLC-procedure (1).

Material and Methods

100 mg of ochratoxin A (Sigma) is dissolved in 100 ml distilled water. This solution is diluted by water to appropriate concentrations for spiking and for checking recovery by the eluent.

Blood: Blood is collected at a slaughterhouse in 1/2 litre portions. To prevent coagulation 32 ml citrate solution is added to each portion. (4 g citric acid, 12.5 g glucosemonohydrate, 11 g sodium citrate and 250 ml distilled water). For a standard curve 50 ml portions of whole blood samples are spiked with ochratoxin solution to give samples with 0, 1, 10, 25 and 50 ppb. Equilibration time is 15 min. at room temperature. Centrifugation for 15 min. at 300 x g. Note the amount of plasma and blood cells, collect 5 ml plasma, add 250 mg celite and 20 ml hydrochloric-magnesiumchloride solution (1:1 0.1 M hydrochloric acid: 0.1 M magnesiumchloride). Adjust pH to 1.8 - 2.0 with 1 M hydrochloric acid. Add 10 ml 2:1 tert. butylacetate:n-hexane, shake for 30 min. and centrifugate for 10 min. at 3000 x g. Shake the tube cautiously by hand to distribute the emulsion layer. Centrifugate for another 5 min. Collect 5 ml organic phase and extract the ochratoxin twice with 3 ml 0.1 M sodiumbicarbonate. Acidify the combined aqueous phase to below pH 2 with formic acid. Back-extract the ochratoxin with 2 x 2 ml chloroform. (It is possible to use 2 x 2 ml tert. butylacetate instead, but recovery tends to decrease). The organic phase is evaporated under nitrogen in a 40°C water bath. (The residue can be stored at -18°C until analysis). The residue is dissolved in 500 µl of eluent.

Tissue: Kidneys used for the standard curve are kidneys without macroscopic changes. Normal kidneys were bought at the local butcher. Abnormal kidneys were received from various Danish slaughterhouses. For a standard curve 20g portions of minced tissue were spiked with ochratoxin A solution to give samples with 0, 5, 10, 20, 25 and 40 ppb. Equilibration time 15 min. at room temperature. Add 100 ml 1:1 isohexane - ethylacetate. (It is also possible to use 100 ml 1:3 n-heptan - ethylacetate for muscle samples) and 20 ml 0.1 m phosphoric acid to the tissue and sonificate (BRANSON 8200) for 15 min. Add 4 g celite and shake for 10 min. Celite binds major cell components and proteins. Adjust pH to approx. 1.5 with 5 M hydrochloric acid. Shake for a few more minutes and centrifugate for 5 min. at 3000 x g.

The supernatant is collected and can be stored overnight at 5°C. 40 ml supernatant is shaken with 8 ml 0.1 M sodium bicarbonate, 4 ml of the aqueous phase is collected and pH adjusted with formic acid to below 2.5. The ochratoxin is back-extracted by 2 x 2 ml tert.butylacetate (shake 2 min.). The organic phase is evaporated under nitrogen in a 60°C water bath. The residue can be stored at -18°C. The residue is dissolved in 400 µl acetonitrile and 400 µl 5% acetic acid is added. Samples are passed through a 0.45 µm PP-PTFE filter (Chromafil from Macherey-Nagel) before injection into the HPLC.

HPLC Conditions:

Eluent: tissue 48:52,; blood 50:50 - acetonitrile: 5% acetic acid

Column: Lichrospher C18 4 mm x 12.5 cm. 5 µm with guard column (Merck)

Sample amount: 20 µl

Equipment: Pump (Perkin Elmer binary 250); Fluorescence detector (Perkin Elmer LC 240); operating at Ex. 330 nm and Em. 460 nm. Data processing by Omega (Perkin Elmer).

Results and Discussion

Chromatograms for blood, muscle and kidney samples are presented in Figures 1, 2 and 3. The A figures represent unspiked samples and the B figures represent known positive samples.

Fig. 1 A
Unspiked blood

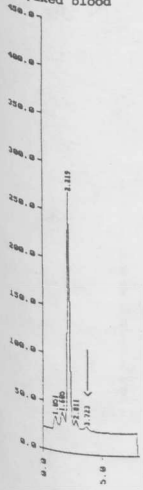


Fig. 1 B
Blood spiked with
25 ppb

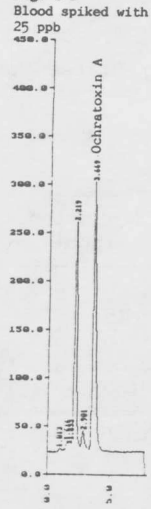


Fig. 2 A
Unspiked muscle

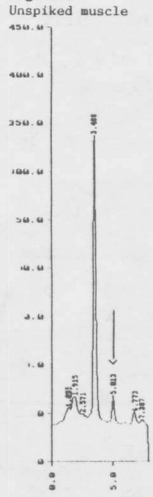


Fig. 2 B
Muscle spiked with
5 ppb

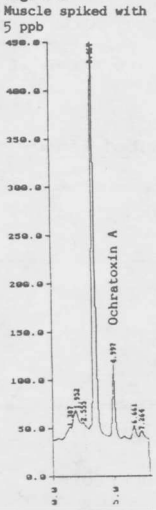


Fig. 3 A
Unspiked kidney

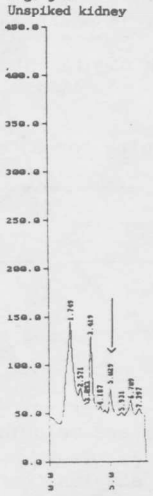
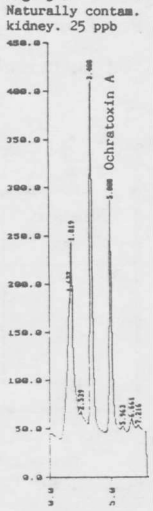


Fig. 3 B
Naturally contam.
kidney. 25 ppb



Obviously there is high background. This could be a natural background, or it could be caused by coeluting components. If it is a coeluting component it will probably not elute with the same retention time in systems with another selectivity. For this reason tissue samples from different pigs and a standard of ochratoxin A dissolved in the eluent are analyzed in 4 different systems.

1. Acetonitrile: 5% acetic acid. Composition 48:52; 50:50
2. Methanol: 5% acetic acid. Composition 66:34; 72:28
3. 2-Propanol: acetonitrile: 0.25 M phosphoric acid: distilled water 20:25:37:18
4. Acetonitrile: 0.25 M phosphoric acid: distilled water 48:35:17

In all systems the small peak from the unspiked sample is eluting at the same retention time as does the ochratoxin peak from the standard and from the spiked samples. From these experiments it is not possible to state that the normally looking kidney and the random blood and muscle samples are not naturally contaminated with ochratoxin A. The peaks of the unspiked samples correspond to 0.5 - 2 ppb for muscle and blood and 0.5 - 3 ppb for kidney. Confirmation by mass spectrometry is preferred. At present such a method is under development. A standard curve for each matrix is shown in Figures 4, 5 and 6 together with the 95% confidence interval.

Figure 4: Standard curve & 95% confidence interval for blood

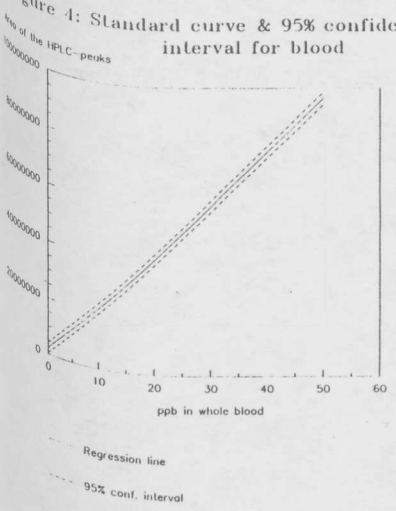


Figure 5: Standard curve & 95% confidence interval for kidney tissue

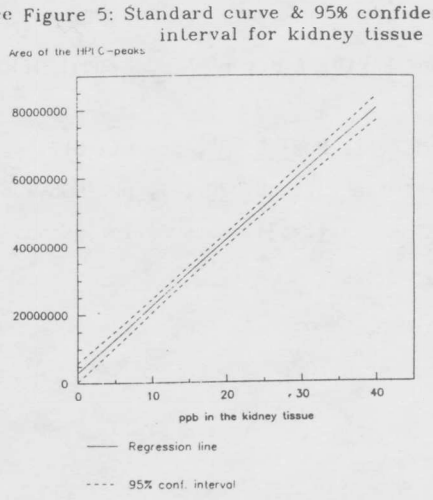
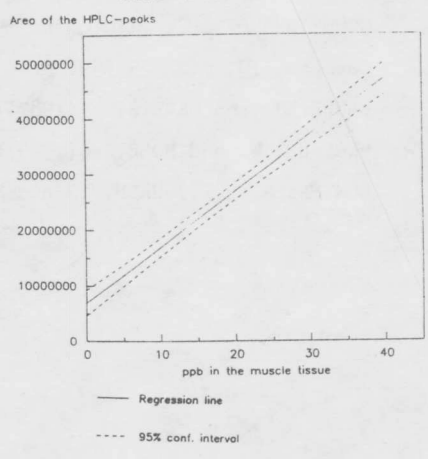


Figure 6: Standard curve & 95% confidence interval for muscle tissue



The method developed gives a linear response for blood between 0 and 50 ppb, and for tissue between 0 and 40 ppb. The correlation coefficient is always better than 0.990. The maximal confidence interval - at the highest and lowest concentration - is not wider than ± 2 ppb for all matrices. Recovery is between 60 and 98% being rather constant within a day.

For kidneys it is possible to compare the results for naturally contaminated abnormal kidneys with the method applied in Denmark at present. A few results are shown in Table 1. More results will be presented at the congress.

Table 1: Results for naturally contaminated kidney samples

Number	Danish control method (1) (ppb)	New method (ppb)
A	<10	<5; <5
B	<10	<5; <5
C	10	6; 6
D	25	26; 31
E	50	>40(50);>40(51)

Numbers in () are calculated by extrapolation.

For these few samples the results are comparable.

Conclusion

A new faster extraction procedure where it is possible to omit chloroform was developed. With HPLC and fluorescence detection a sufficient standard curve and very tight 95% confidence intervals are obtained. For kidney samples the method tends to give similar results as the present method used in Denmark for control (1). It is necessary to perform further tests on naturally contaminated samples for all matrices. Because of the high background the results of the HPLC-runs should in addition be compared to MS-analysis for confirmation.

References

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