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Determination of Aflatoxin B_1-Lysine in Pig Serum and Plasma by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

Aflatoxin B_1 (AFB_1) is a hepatocarcinogen produced by certain *Aspergillus* species growing on crops. After biotransformation in the liver, AFB_1 generates several metabolites, one of which is AFB_1 bound to lysine on serum albumin. AFB_1-lysine (AFB_1-lys) is a digest product of AFB_1-albumin and is considered a biomarker of exposure to AFB_1 in humans and animals. The objectives of this paper were to evaluate the performance characteristics of a new analytical method for determination of AFB_1-lys levels in pig serum, heparinized and ethylenediaminetetraacetic acid (EDTA) plasma and to evaluate the interference of these anticoagulants in AFB_1-lys quantification. Blank blood samples were obtained from eight crossbreed 91-day-old barrows fed AFB_1-free diets. Pooled samples ($n = 3$) and individual samples of serum, EDTA and heparinized plasma collected from five pigs were enzymatically digested with pronase at 37°C for 4 h. AFB_1-lys was isolated by solid-phase extraction and quantified by liquid chromatography coupled to tandem mass spectrometry. The analytical method was applied for determination of AFB_1-lys in serum and EDTA plasma collected from five 49-day-old crossbreed barrows fed *ad libitum* diets containing 1.1 mg of AFB_1 per kg of feed during 7 days (three animals) or 42 days (two animals). Samples of heparinized plasma were only available from animals intoxicated for 42 days. All animals had lower levels of AFB_1-lys in EDTA plasma samples (24.78–37.40 ng/mL), when compared to serum (49.32–252.07 ng/mL^{-1}) or heparinized plasma (176.81 and 264.24 ng/mL^{-1}). EDTA did not interfere in AFB_1-lys standard detection, but our findings suggest that EDTA should be avoided during blood collection since it affects the pronase activity in AFB_1–albumin adduct digestion and, consequently, causes a reduction in the AFB_1-lys levels. Hence, determination of AFB_1-lys in serum and heparinized plasma is an approach to assess an individual's exposure of swine to AFB_1.

Introduction

Aflatoxins are secondary metabolites produced by fungi of the genus *Aspergillus*, mainly *A. flavus*, *A. parasiticus* and *A. nomius* (1). Aflatoxin B_1 (AFB_1) is the most abundant, being highly toxic, mutagenic and hepatocarcinogenic. These molds are frequently found on foodstuffs, particularly maize and ground nuts, resulting in human and animal exposure (2). Susceptibility to the toxic effects of AFB_1 varies with age, aflatoxin concentration and duration of exposure, as well as other factors (3). The primary target organ is the liver, which is also the main organ where AFB_1 undergoes biotransformation by liver cytochrome P450 enzymes. The most important product of AFB_1 biotransformation is AFB_1-8,9-epoxide, which bonds covalently to DNA guanine residues and serum albumin (4, 5). The aflatoxin B_1-lysine (AFB_1-lys) adduct is a digestion product of AFB_1–albumin; thus, the measurement of AFB_1-lys in human blood (often expressed as AFB_1-lys/mg albumin) has been used as a valuable biomarker of long-term exposure (2, 3) to AFB_1 in the diet (6).

The exposure of livestock to mycotoxins has traditionally been investigated by analyzing food ingredients (7). However, this approach has important limitations such as the inability to measure individual animal exposure and the high variability of results due to variations of mycotoxin contamination in the feed. In recent years, analytical methods for the determination of biomarkers of several mycotoxins in pig plasma have been developed (7–9), but levels of AFB_1-lys have not been published. For AFB_1-lys analysis in human serum and plasma, samples are usually digested with pronase for 4–5 h at 37°C (10, 11), but the digestion kinetics for serum or plasma from livestock has not been reported.

Blood collection plays an important role in investigations of mycotoxicosis outbreaks in livestock or in experimental trials (12). Blood products obtained with clotting (serum) or with anticoagulant addition (plasma) can differ in terms of metabolite levels, which can be attributed to different mechanisms of clotting and anticoagulation processes (13). In addition, metabolic profiles may be altered in plasma depending on the type of anticoagulant (13). Heparin is the most widely used anticoagulant for biochemical analysis and can be used to evaluate hematological parameters. However, blood samples collected with ethylenediaminetetraacetic acid (EDTA) are preferable for hematological examination. While heparin inhibits thrombin formation, EDTA works by chelating calcium ions thus preventing coagulation of proteins (13). However, the presence of anticoagulants can cause interferences with components of the sample, such as enzyme inhibitors, fibrinogen and cations (14). The interference of anticoagulants in blood clinical chemistry analysis has been recently reported in several studies (13–18). However, there is no previous study evaluating possible interferences of these anticoagulants on analytical methods for AFB_1-lys in plasma. Therefore, the aim of this study was to evaluate the performance characteristics of an analytical method for determination of AFB_1-lys levels in individual and pooled samples of pig serum, heparinized and EDTA plasma using Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS), and apply the method to quantitate AFB_1-lys in samples from AFB_1 exposed pigs.

Experimental

Chemicals and reagents

AFB_1-lys standard was synthesized, purified and characterized as originally described by Scholl and Groopman (19) with modifications proposed by Sass *et al.* (20). AFB_1 was purchased from Sigma (St. Louis, MO, USA). Pronase (64 kU/g) was purchased from Calbiochem (Merck®, Darmstadt, Germany). Mixed-mode solid-phase extraction (SPE) cartridges (Oasis MAX, 1 cm^3, 30 mg) were purchased from Waters Corp. (Milford, MA, USA). Methanol, acetonitrile and ammonium hydroxide solution (25%) were purchased from Merck®. Formic acid was purchased from J.T.Baker® (Xalostoc, Mexico). Milli-Q water was produced by a Millipore system (Millipore Corporation, Billerica, MA, USA).

Blood samples

Because certified blank blood samples were not available, samples with undetected levels of AFB_1-lys, collected from eight crossbreed 91-day-old barrows (47.28 ± 4.33 kg) fed AFB_1-free diets, were chosen as "blank" samples and used in spiking and recovery studies. After fasting for 12 h, blood samples were collected via jugular venipuncture from pigs in three different types of evacuated Vacuette® tubes (Greiner Bio-one, Kremsmunster, Austria): serum separator clot activator tube, sodium heparin tube and K3 EDTA tube. Serum samples were allowed to clot for ≥30 min at room temperature and then, both serum and plasma samples were centrifuged at 2,950 *g* for 10 min. The samples collected from three blank animals were mixed to form a pool of serum, EDTA and heparinized plasma samples, which were aliquoted into 2 mL micro tubes and submitted to AFB_1-lys analysis. The remaining individual blank samples of serum, EDTA and heparinized plasma ($n = 5$ of each type) were analyzed individually. All samples were stored at −80°C until analysis.

Evaluation of method performance characteristics

The performance characteristics of the analytical method for AFB_1-lys in serum, EDTA and heparinized plasma were evaluated by using pooled blank samples from pigs fed AFB_1-free diets fortified with the AFB_1-lys standard at 10.3, 20.6, 41.2, 82.5, 165.0 and 330.0 ng/mL. Additionally, the analytical method for AFB_1-lys was further evaluated by using the five individual blank samples fortified with the AFB_1-lys standard at 20.6, 41.2, 82.5, 165.0 and 330.0 ng/mL. Parameters evaluated included linearity, limit of determination (LOD), limit of quantification (LOQ), recovery and matrix effect (21). Fortified serum and plasma samples were submitted to enzymatic digestion and extraction procedures for AFB_1-lys analyses according to McCoy *et al.* (2) with minor modifications. Triplicate aliquots (250 µL) of serum, EDTA and heparinized plasma were placed in 2 mL micro tubes, mixed with pronase solution (250 µL, 13 mg/mL) and incubated in a water bath at 37°C for 4 h. Sequentially, Milli-Q water (500 µL) was added to each sample and samples were loaded onto Oasis MAX SPE extraction cartridges preconditioned with MeOH (1 mL) and water (1 mL). Cartridges were washed with water (2 × 1 mL), 70% MeOH:water (*v/v*, 1 mL), freshly prepared 1% NH_4OH in MeOH (*v/v*, 1 mL) and MeOH (0.5 mL). AFB_1-lys was eluted with freshly prepared 2% formic acid; MeOH (*v/v*, 1 mL) and evaporated to dryness in a rotational vacuum concentrator (MartinChrist, Osterode am Harz, Germany) at 0.01 mBar and 40°C for 2 h. Final extracts were resuspended in 0.2 mL 25% MeOH:water (*v/v*).

LC-MS/MS analysis of AFB_1-lys

Resuspended extracts were injected into a Waters Acquity Class-1 ultra-performance liquid chromatographic (UPLC) system coupled to a Xevo TQ-S® mass spectrometer (Waters) operated in the positive ionization electrospray mode. The auto injector tray

temperature was maintained at 15°C, and the injection volume was 10 µL. The column utilized was a BEH C_{18} (2.1 × 50 mm, 1.7 µm) column and the mobile phase consisted of (A) H_2O/0.1% formic acid (*v/v*) and (B) acetonitrile/0.1% formic acid (*v/v*). The gradient profile was for 0–0.50 min (95% A, 5% B), 0.50–5.00 min stepped to 75% A and 25% B; 5.00–5.50 (10% A and 90% B) followed by 95% A and 5% B held until the end of the run at 6 min. The flow rate was 500 µL/min. AFB_1-lys was detected in the Multiple Reaction Monitoring (MRM) scan mode at 30 V cone energy and 38 V collision energy for all *m/z* transitions. The monitored transitions were 457.16→394.0, 457.16→376.00, 457.16→348.00 and 457.16→328.00. Quantification and confirmatory MRM transition for AFB_1-lys were *m/z* 457→394 and 457→348, respectively. Ion ratio between quantification and confirmatory transition (*m/z* 457→394 / 457→348) was calculated as 1.5 in pure solvent, and 1.3 in the matrices of serum, EDTA or heparinized plasma. Our tolerance was ±20%. The parameters of the mass spectrometer such as the capillary voltage (0.75 kV), desolvation temperature (650°C), desolvation gas flow (500 L/h), nebulizer gas flow (5.0 bar), cone gas flow (150 L/h) and source temperature (150°C) had been previously optimized. A working solution of AFB_1-lys standard (1.0 µg/mL) was prepared in 25% methanol and diluted to 10.3, 20.6, 41.2, 82.5, 165.0 and 330.0 ng/mL. AFB_1-lys standard solutions were prepared in triplicate and analyzed by LC-MS/MS to generate a 5-point standard calibration curve. The LOD and LOQ values were calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively. Linearity was evaluated by verifying the coefficient of determination (r^2).

Standard calibration curves (linear, 1/*x* weighted) for AFB_1-lys were constructed by plotting the peak area versus the analyte concentration. In the same way, the MS peak areas obtained from spiking samples before and after extraction were plotted against the actual spiking levels. The equations of apparent recovery (R_A) (Equation (1)), signal suppression/enhancement (SSE) due to matrix effects (Equation (2)) and recovery of the extraction step (R_E) (Equation (3)) were calculated according Sulyok *et al.* (21) and Warth *et al.* (22), as follows:

$$R_A(\%) = 100^* \text{ slope}_{\text{spiked sample}} / \text{slope}_{\text{liquid standard}} \quad (1)$$

$$SSE(\%) = 100^* \text{ slope}_{\text{spiked extract}} / \text{slope}_{\text{liquid standard}} \quad (2)$$

$$R_E(\%) = 100^* R_A / SSE \quad (3)$$

The R_A value expresses the ratio of an observed value obtained from a calibration curve divided by a reference known or theoretical value. R_E is expressed as the yield of a pre-concentration or extraction stage divided by the amount of analyte in the original sample. In this paper, R_A is composed multiplicative by R_E and SSE (21).

Analyses of pig serum, EDTA and heparinized plasma

The analytical method was applied for determination of AFB_1-lys levels in serum and EDTA plasma collected from five 49-day-old crossbreed barrows (15.5 ± 1.2 kg) fed *ad libitum* diets containing 1.1 mg of AFB_1 per kg of feed during 7 days (three animals) or 42 days (two animals). Samples of heparinized plasma were only available from animals intoxicated for 42 days. Blood sample collection was conducted after fasting for 12 h, and AFB_1-lys analyses were performed in duplicate samples as previously described. Additionally, the enzymatic protein digestion was evaluated in one of the animals, from which a sufficient volume of serum, EDTA and heparinized samples were collected to assess the AFB_1-lys concentrations in triplicate assays after 4-, 8-, 12- and 16-h incubation in a water bath at 37°C.

Statistical analysis

The analytical results were subjected to ANOVA using GraphPad Prism, and variable means showing significant differences were compared using the Tukey's multiple comparisons test (23). All statements of significance are based on the 0.05 level of probability.

Results

Method performance

Figure 1A presents the chromatogram obtained from AFB_1-lys standard (330 ng/mL) at retention time of 3.17 min. The performance results obtained in pooled samples of serum, EDTA and heparinized plasma are presented in Table I. R_A, SSE and R_E values among the different matrices analyzed varied from 21.92% to 22.30%, 31.14% to 37.39% and 57.58% to 69.47%, respectively. The levels of AFB_1-lys detected in serum, EDTA and heparin plasma fortified before the digestion process were significantly suppressed in all levels of the calibration curve, compared with the standard curve in solvent (R_A) and fortified extract (SSE). R_E values were more than two times higher than R_A (Table I), indicating a significant matrix suppression of the analyte response. Therefore, matrix matched calibration curves should be used to perform a reliable quantitative analysis of AFB_1-lys. In order to simulate the same process that the serum and plasma samples underwent, all the standards used for preparation of calibration curves to quantify the samples were fortified before the extraction process. In addition, for the concentration range employed (10.3–330.0 ppb), a satisfactory linearity was achieved ($r^2 > 0.99$) for all matrices. The LODs and LOQs of AFB_1-lys in all matrices varied between 2.69–3.38 ng/mL and 9.01–10.31 ng/mL, respectively. The coefficient of determination did not show any tendency or deviation from linearity.

Table II presents the performance parameters for determination of AFB_1-lys in individual samples of serum, EDTA and heparinized plasma from five different pigs. R_A, SSE and R_E values varied from 22.97% to 23.60%, 32.32% to 39.04% and 58.83% to 72.39%, respectively. Satisfactory linearity values ($r^2 > 0.99$) were also obtained for all matrices at the concentration range of 20.6–330.0 ng/mL, and LODs and LOQs of AFB_1-lys ranged from 2.91 to 3.77 ng/mL and 9.84 to 11.99 ng/mL, respectively

Dosimetry of AFB_1-lys in AFB_1-dosed pigs

Only the animals fed AFB_1-contaminated diets for 42 days showed clinical signs of typical aflatoxicosis, such as apathy, decrease in feed intake and body weight. The results of AFB_1-lys concentrations of AFB_1-dosed pigs are presented in Table III. AFB_1-lys levels were higher in the two pigs fed AFB_1 for 42 days, compared to the three animals intoxicated for 7 days. All animals had much lower levels of AFB_1-lys ($P > 0.05$) in EDTA plasma samples than in serum or heparinized plasma, and no difference ($P > 0.05$) was found between these two types of samples from pigs fed AFB_1 for 42 days. Figure 1B presents the total ion chromatogram of the AFB_1-lys isolated in serum from one of the dosed pig, which contained the highest level of the compound (252.07 ng/mL). In the pronase digestion experiment, serum and heparinized plasma also had

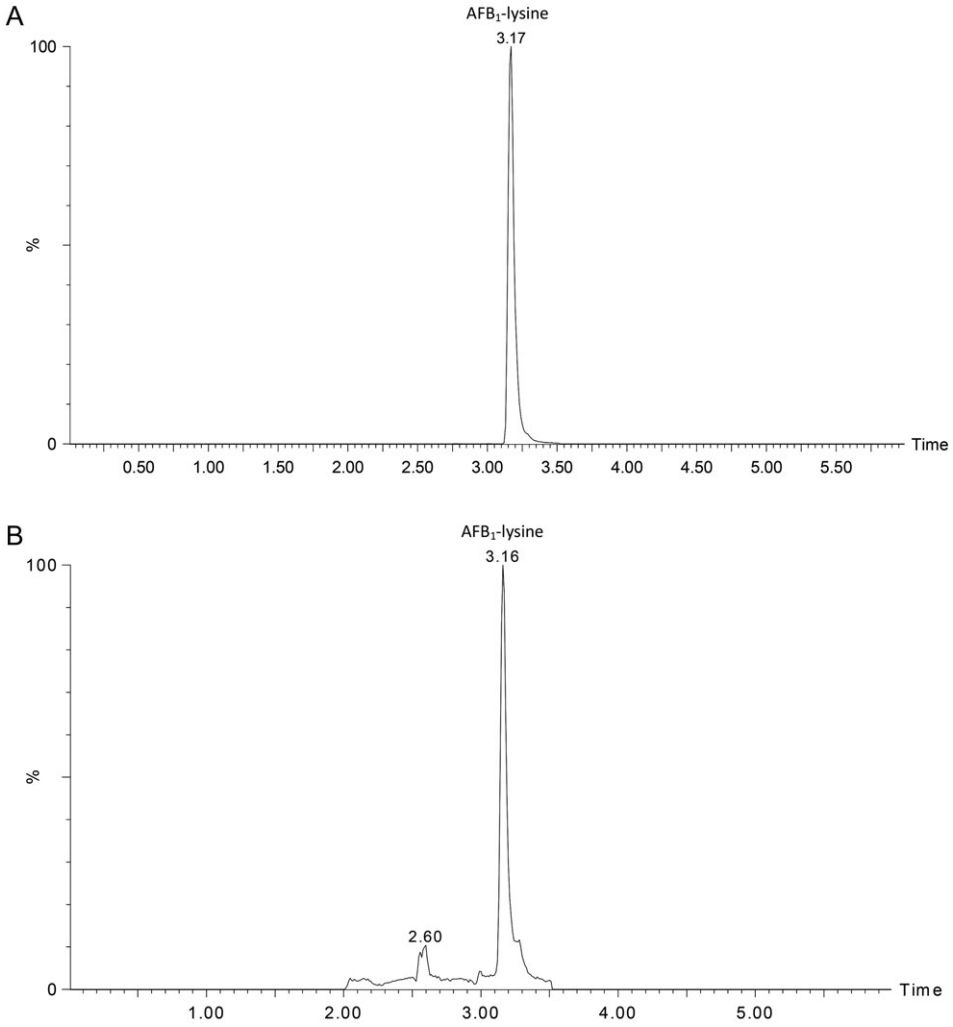

Figure 1. Total ion current chromatograms of AFB_1-lys from a blank pig sample spiked with 330 ng/mL of AFB_1-lys standard (A) and from a serum sample of pig dosed with AFB_1, containing 252.07 ng/mL of AFB_1-lys (B).

Table I. Method performance parameters for determination of AFB_1-lys in pooled samples ($n = 3$) of pig serum, EDTA and heparinized plasma

Matrix	Conc. range ($ng.mL^{-1}$)	r^2	R_A (%)	SSE (%)	R_E (%)	LOD (ng/mL)	LOQ (ng/mL)	RSD (%) low/high	CV (%)
Serum	10.3–330.0	0.9969	21.92	37.39	57.58	2.69	9.01	4.4/2.1	4.2
EDTA plasma	10.3–330.0	0.9964	22.27	31.14	69.47	3.09	10.31	4.8/1.6	4.6
Heparinized plasma	10.3–330.0	0.9959	22.30	33.23	65.89	3.38	11.00	6.0/2.4	6.4

RSD, relative standard deviation at the lowest and the highest concentration levels; CV, coefficient of variation.

Table II. Method performance parameters for determination of AFB_1-lys in individual samples of pig serum, EDTA and heparinized plasma ($n = 5$)

Matrix	Conc. range ($ng.mL^{-1}$)	r^2	R_A (%)	SSE (%)	R_E (%)	LOD (ng/mL)	LOQ (ng/mL)	RSD (%) low/high	CV (%)
Serum	20.6–330.0	0.9907	22.97	39.04	58.83	2.91	9.84	5.9/3.3	5.8
EDTA plasma	20.6–330.0	0.9971	23.40	32.32	72.39	3.77	11.99	6.9/2.9	5.4
Heparinized plasma	20.6–330.0	0.9927	23.60	36.31	64.89	3.59	11.62	7.2/3.2	7.3

RSD, relative standard deviation at the lowest and the highest concentration levels; CV, coefficient of variation.

Table III. AFB_1-lys levels in serum, EDTA and heparinized plasma samples of pigs intoxicated with AFB_1 for 7 or 42 days[a]

Animal	Days of intoxication	Serum (ng/mL)	Heparinized plasma (ng/mL)	EDTA plasma (ng/mL)
1	7	49.32 ± 4.05[b]	ND	24.98 ± 0.78[b]
2	7	59.82 ± 3.33[b]	ND	27.86 ± 1.52[b]
3	7	74.24 ± 6.49[b]	ND	27.97 ± 2.63[b]
4	42	190.62 ± 7.28[b]	176.81 ± 16.64[b]	37.40 ± 3.85[b]
5	42	252.07 ± 10.08[b]	264.24 ± 23.57[b]	24.78 ± 1.47[b]

[a]Results are reported as mean ± SD for triplicate analyses.
[b]In the same row, means with different superscript letters differ significantly ($P < 0.05$).
ND, not determined.

similar ($P > 0.05$) levels of AFB_1-lys from the hydrolysis of the AFB_1–albumin at all times tested (4–16 h); although EDTA plasma samples had increased levels of AFB_1-lys with increasing digestion times, but at concentrations were much lower ($P < 0.05$) than those observed in serum and heparinized plasma in all time periods evaluated (data not shown).

Discussion

Including all species, this is the first report on comparison of matrix effects related to serum, EDTA and heparinized plasma samples in AFB_1-lys quantification. The enzymatic digestion used in our experiment (4 h) was based on previous studies with pronase and heparinized plasma or serum samples from rats and humans (2, 24–26).

As shown in Table I, the values of R_A, R_E and SSE of pooled EDTA plasma and other matrices were similar. Taking into account that the extraction and LC-MS/MS procedures for AFB_1-lys analyses were performed according to a previously described method for human samples (2), with minor modifications, the performance results indicate good reliability of the method for determination of AFB_1-lys in pig serum and plasma. R_A, SSE and R_E values obtained for individual samples of serum, EDTA or heparinized plasma (Table II) were also similar to those obtained for each respective matrix from pooled samples (Table I), hence indicating low variability of matrix effect among samples from different animals. The animals used in the present study were all crossbreed and received the same AFB_1-free diet for several weeks before blood sample collection. Thus, similar blood chemical composition was expected to occur among those pigs, which may have contributed for the low variability of matrix effect for samples from different individuals. It remains to be determined if samples from pigs from different lineages or receiving different diets would result in higher variability of matrix effects in the AFB_1-lys analyses.

The higher AFB_1-lys concentrations found in pigs fed AFB_1 for 42 days, compared to animals intoxicated for 7 days (Table III), are consistent with data describing the dose-dependent temporal increase in the AFB_1-lys levels after continuous administration of AFB_1 (24). However, the levels of AFB_1-lys found in EDTA plasma were much lower than serum or heparinized plasma samples evaluated. EDTA did not interfere in AFB_1-lys standard detection, but our findings suggest that EDTA affects the pronase activity in AFB_1–albumin adduct digestion and, consequently, causes a reduction in the levels of AFB_1-lys regardless of the digestion time used.

Walker and Sweeney (27) reported that pronase is composed of a group of proteolytic enzymes (containing at least 10 proteolytic components) and is used in cases where extensive or complete degradation of protein is required. The dependence of calcium ion for the stability of some components was one of the earliest observations made of pronase. Irreversible loss of 70% of proteolytic activity may happen if EDTA was added in excess (27). This effect may be related to the mechanism of EDTA activity in coagulation, by chelating the calcium of a blood sample, which is in accordance with the results reported in this paper (27). Additionally, these findings are also important for the interpretation of human AFB_1-lys data, since previous studies have used EDTA plasma samples to detect AFB_1–albumin adducts in human samples (28–31). Concentrations of AFB_1-lys in EDTA plasma samples may appear to be negative or underestimated. Moreover, there are other reports of AFB_1-lys that do not specify the anticoagulant used to obtain the plasma sample (32–34). Another point to consider is that the results containing EDTA can only be compared with data that used the same matrix.

Conclusion

The analytical method described was evaluated and successfully applied in samples from pigs fed with AFB_1. Both serum and heparinized plasma are good options of matrices for evaluating AFB_1-lys levels in pig samples for diagnostic purposes, and their results can be compared. However, EDTA plasma is not recommended, because EDTA may affect pronase digestion, thus decreasing the level of AFB_1-lys that can be detected. Hence, the determination of AFB_1-lys in serum and heparinized plasma is an approach to assess an individual's exposure of swine to AFB_1.

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