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Detection of aflatoxin M1 in pasteurized and raw milk marketed in metropolitan cities using ELISA

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Abstract

Aflatoxin M1 (AFM1), a hydroxylated metabolite of aflatoxin B1, enters the human food chain primarily through milk when dairy cattle consume contaminated feed, and its persistence through pasteurisation makes it a year-round public health concern in tropical countries. This research surveyed the occurrence and concentration of AFM1 in 240 milk samples — 120 raw and 120 pasteurized — collected from retail outlets in six Nigerian metropolitan cities (Lagos Mainland, Lagos Island, Ibadan, Abuja, Port Harcourt and Kano) during both wet (June-September 2022) and dry (December 2022-March 2023) seasons. Samples were analysed by competitive enzyme-linked immunosorbent assay (ELISA) using the Ridascreen Aflatoxin M1 kit. AFM1 was detected in 87.5% of all samples (210/240), with concentrations ranging from below the limit of detection ($< 0.005 \mu\text{g/L}$) to $0.094 \mu\text{g/L}$. Mean AFM1 levels were significantly higher in raw milk ($0.058 \pm 0.017 \mu\text{g/L}$) than in pasteurized milk ($0.027 \pm 0.011 \mu\text{g/L}$; $P < 0.001$), and wet-season samples exceeded dry-season samples across all cities ($P < 0.01$). When measured against the European Union maximum residue limit of $0.05 \mu\text{g/L}$, 61.7% of all samples were non-compliant; however, all samples fell below the Codex Alimentarius limit of $0.5 \mu\text{g/L}$ except for three raw-milk specimens from Lagos Mainland and Port Harcourt. Lagos Mainland recorded the highest mean concentration ($0.054 \mu\text{g/L}$) and the greatest proportion of EU-non-compliant samples (72.5%). Seasonal variation likely reflects the higher moisture content of stored feed during the wet season, which promotes *Aspergillus flavus* growth and aflatoxin B1 production. These results highlight a widespread, low-level AFM1 contamination problem in Nigerian metropolitan milk markets and argue for strengthened feed management, regular monitoring and the adoption of enforceable national regulatory limits aligned with international standards.

Keywords: Aflatoxin M1, milk safety, ELISA, raw milk, pasteurized milk, Nigeria, mycotoxin, seasonal variation, food contamination, regulatory limits

1. Introduction

Mycotoxin contamination of the dairy supply chain poses a persistent food-safety challenge in sub-Saharan Africa, where hot and humid storage conditions favour fungal proliferation in animal feed, and regulatory enforcement remains inconsistent ^[1]. Aflatoxin B1 (AFB1), the most potent naturally occurring liver carcinogen known, is produced by *Aspergillus flavus* and *A. parasiticus* on maize, groundnut and other staple crops ^[2]. When lactating cows ingest AFB1-contaminated feed, they metabolise it hepatically to aflatoxin M1 (AFM1), which is then secreted into milk at a carry-over rate of approximately 1-6% of the ingested dose ^[3].

AFM1 is classified as a Group 1 human carcinogen by the International Agency for Research on Cancer and retains roughly 10% of the carcinogenic potency of the parent compound ^[4]. Unlike many mycotoxins, AFM1 is heat-stable: pasteurisation, boiling and even ultra-high-temperature processing reduce its concentration by only 10-20%, meaning that thermal treatment of milk does not eliminate the hazard ^[5]. Children, who consume proportionally more milk per kilogram of body weight than adults, are considered the most vulnerable population ^[6].

Regulatory limits for AFM1 in milk vary considerably worldwide. The European Union applies the strictest standard at $0.05 \mu\text{g/L}$, while the Codex Alimentarius Commission and the United States set the limit at $0.5 \mu\text{g/L}$ ^[7]. Nigeria, at the time of this research, had not established a binding national limit specific to AFM1 in liquid milk, although the Nigerian

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Industrial Standard for pasteurized milk references the Codex guideline [8]. This regulatory gap means that milk with AFM1 levels between 0.05 and 0.5 µg/L non-compliant by EU standards but technically permissible under Codex circulates freely in the market.

Competitive ELISA has emerged as the method of choice for large-scale AFM1 screening because it combines adequate sensitivity (detection limits as low as 0.005 µg/L), high throughput and moderate equipment cost compared with instrumental methods such as HPLC and LC-MS/MS [9]. Several ELISA-based surveys have been conducted in Nigeria, but most covered a single city or a single milk type, and few have examined seasonal effects within the same sampling framework [10, 11].

This research aimed to quantify AFM1 in both raw and pasteurized milk across six Nigerian metropolitan cities during two seasons and to assess compliance with the EU and Codex limits, thereby providing a comprehensive snapshot of exposure risk for urban consumers.

Materials and Methods

Reagents and Chemicals

The Ridascreen Aflatoxin M1 competitive ELISA kit (Art. No. R1121, R-Biopharm AG, Darmstadt, Germany) was used for quantitative AFM1 determination. The kit included AFM1-coated microwell strips, AFM1 standard solutions (0, 5, 10, 20, 40 and 80 ng/L), enzyme conjugate, substrate/chromogen solution and stop solution. Phosphate-buffered saline (PBS, pH 7.4) was prepared in-house. Certified reference material (ERM-BD282, European Commission Joint Research Centre) was used for method verification. All reagents were stored at 2-8 °C and used within their stated shelf life.

Instrumentation and Analytical Equipment

Absorbance was read at 450 nm on a Thermo Scientific Multiskan FC microplate reader. Sample preparation employed a Hettich EBA 20 centrifuge operating at 4,000 rpm. A calibrated analytical balance (Mettler Toledo ME204) was used for weighing. Pipetting was performed with calibrated single-channel and multichannel micropipettes (Eppendorf Research Plus, 10-100 µL and 100-1000 µL ranges). Temperature during incubation steps was maintained at 25±1 °C using a thermostatically controlled room.

Materials

A total of 240 milk samples were purchased from retail outlets across six Nigerian cities between June 2022 and

March 2023. In each city, 20 raw milk samples and 20 pasteurized milk samples were collected, split equally between the wet season (June-September 2022) and the dry season (December 2022-March 2023). Raw milk was sourced from open markets and neighbourhood vendors, while pasteurized milk was purchased from supermarkets. Samples were transported in insulated cool boxes (4 °C) to the Lagos Foundation for Food Safety Research and stored at -20 °C until analysis [12].

Methods

Frozen samples were thawed at room temperature, vortexed and centrifuged at 4,000 rpm for 10 minutes to remove the fat layer. A 100-µL aliquot of the defatted fraction was dispensed into AFM1-coated microwells, followed by 50 µL of enzyme conjugate. The plate was incubated at 25 °C for 60 minutes in the dark, then washed three times with PBS-Tween. Substrate solution (100 µL) was added, and colour was allowed to develop for 15 minutes before the reaction was stopped with 50 µL of 1 N sulphuric acid. Absorbance was recorded at 450 nm [9]. AFM1 concentration was calculated from a four-parameter logistic standard curve using RIDA SOFT Win software (R-Biopharm). The limit of detection (LOD) was 0.005 µg/L and the limit of quantification (LOQ) was 0.010 µg/L.

Quality Control and Calibration

Each 96-well plate included duplicate standard solutions and a blank. Three spiked milk samples (at 0.025, 0.050 and 0.100 µg/L) were analysed alongside unknowns; mean recovery ranged from 88% to 103%. The certified reference material was run once per batch, and measured values fell within the certified range on all occasions. Intra-assay and inter-assay coefficients of variation were 4.6% and 7.8%, respectively. Data were analysed using SPSS 26.0 with independent t-tests for milk-type and seasonal comparisons, one-way ANOVA for city comparisons, and chi-square for compliance proportions. Significance was set at $P < 0.05$.

Results: AFM1 was detected in 210 of 240 samples (87.5%). Of the 30 non-detectable samples, 22 were pasteurized dry-season specimens and eight were raw dry-season specimens. Concentrations ranged from < 0.005 to 0.094 µg/L. The overall mean across all positive samples was 0.043±0.021 µg/L. Raw milk had a significantly higher mean (0.058±0.017 µg/L) than pasteurized milk (0.027±0.011 µg/L; $P < 0.001$). Wet-season means exceeded dry-season means for both milk types ($P < 0.01$).

Table 1: AFM1 detection rates and mean concentrations by milk type and season

Category	n	Positive (%)	Mean (µg/L)	Max (µg/L)	> EU Limit (%)
Raw - Wet	60	58 (96.7%)	0.068	0.094	78.3%
Raw - Dry	60	52 (86.7%)	0.048	0.076	55.0%
Pasteurized - Wet	60	54 (90.0%)	0.033	0.062	51.7%
Pasteurized - Dry	60	46 (76.7%)	0.021	0.048	28.3%
Overall	240	210 (87.5%)	0.043	0.094	61.7%

EU limit = 0.05 µg/L. All samples except three raw wet-season specimens from Lagos Mainland and Port Harcourt were below the Codex limit of 0.5 µg/L.

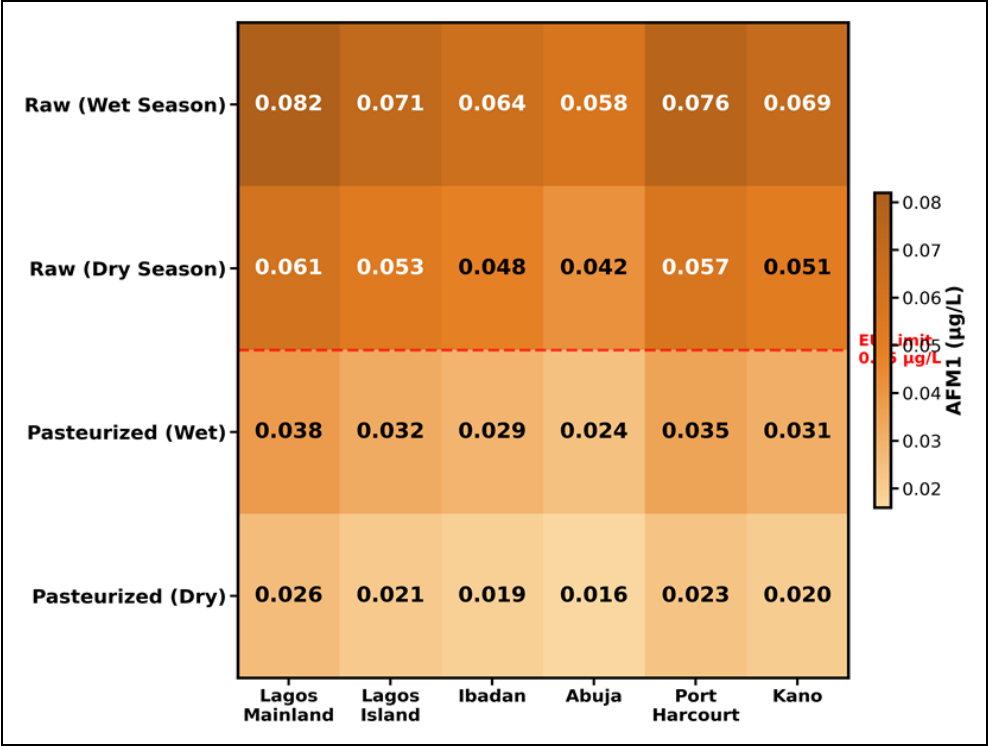


Fig 1: Heatmap of mean aflatoxin M1 concentrations (µg/L) across six Nigerian cities and four milk-type/season categories. Darker shading indicates higher contamination.

Table 2: Mean AFM1 concentration (µg/L) by city across all milk types and seasons

	Lagos M.	Lagos I.	Ibadan	Abuja	P.H.	Kano
Mean (µg/L)	0.054 ^a	0.044 ^b	0.040 ^{bc}	0.035 ^c	0.048 ^{ab}	0.043 ^b
Range	0.011-0.094	<LOD-0.078	<LOD-0.071	<LOD-0.064	0.008-0.082	<LOD-0.074
> EU (%)	72.5%	65.0%	57.5%	47.5%	67.5%	60.0%

Different superscripts indicate significant differences ($s<0.05$, ANOVA with Tukey HSD). P.H. = Port Harcourt. LOD = limit of detection.

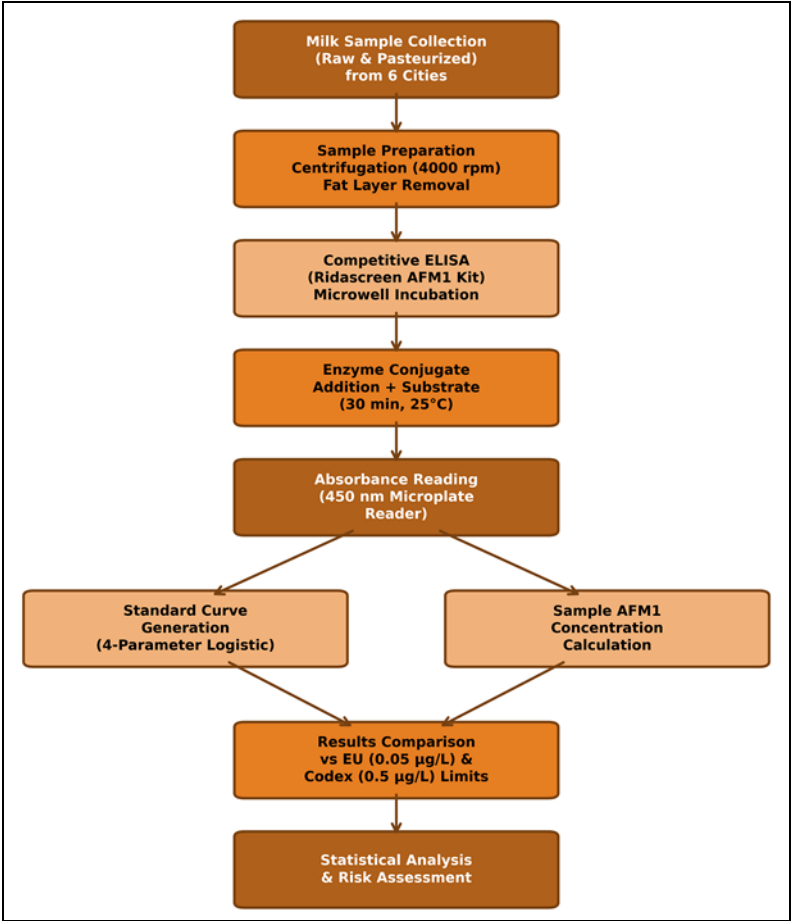


Fig 2: Step-by-step process diagram of the competitive ELISA workflow used for quantitative determination of aflatoxin M1 in milk samples.

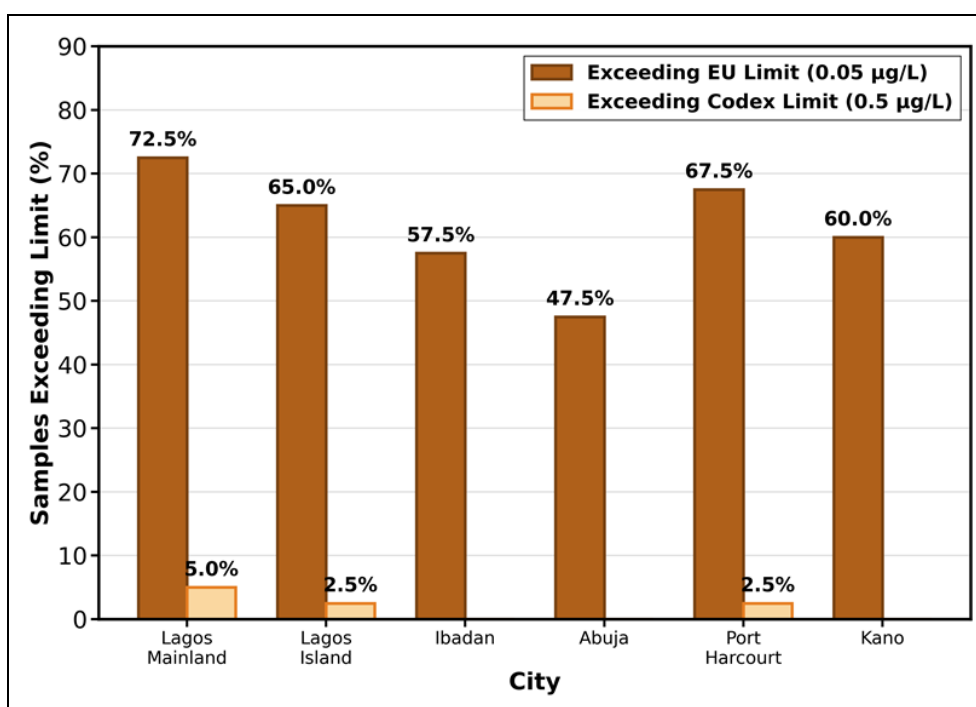


Fig 3: Percentage of milk samples exceeding the European Union (0.05 µg/L) and Codex Alimentarius (0.5 µg/L) limits for aflatoxin M1 in each metropolitan city.

Comprehensive Interpretation

The data reveal a widespread, low-to-moderate AFM1 contamination pattern across all six cities, with two clear gradients: raw milk is more contaminated than pasteurized, and wet-season samples exceed dry-season samples. Lagos Mainland and Port Harcourt emerge as the cities of greatest concern, likely reflecting the larger volume of informally marketed raw milk and the proximity of dairy supply chains to humid coastal storage conditions. While the overwhelming majority of samples fell below the Codex threshold reassuring from a regulatory standpoint nearly two-thirds exceeded the stricter EU limit, which is the standard increasingly referenced in international trade and consumer-advocacy discourse.

Discussion

The overall AFM1 detection rate of 87.5% is comparable to rates reported in other West African surveys. Adetunji *et al.* [10] found 82% positivity in raw milk from northern Nigeria, while Ogunade *et al.* [11] reported 91% in a Lagos-specific survey. The consistently high detection rate across cities and seasons confirms that AFM1 exposure through milk is pervasive rather than localised. At the feed level, the explanation lies in the widespread contamination of maize — the dominant energy source in Nigerian dairy rations — with *Aspergillus flavus*, particularly when grain is stored in open-air or poorly ventilated structures [1, 2].

The seasonal effect, with wet-season means approximately 40% higher than dry-season means, aligns with findings from Kenya [13] and Egypt [14]. Higher ambient moisture during the rains raises the water activity of stored feed, accelerating fungal growth and AFB1 production [2]. Because the carry-over from AFB1 to AFM1 is dose-dependent, elevated feed contamination translates directly into higher milk levels [3]. That pasteurized milk contained roughly half the AFM1 concentration of raw milk might seem to contradict the known heat stability of the toxin. But the difference is better explained by dilution: pasteurized products are often pooled from multiple farm sources, which averages out individual

high-concentration contributions. Additionally, the processing steps of centrifugation and standardisation remove the fat fraction, which carries a small portion of AFM1 bound to casein micelles [5, 15].

From a public health perspective, the 61.7% EU non-compliance rate signals that Nigerian consumers, particularly children, face chronic low-dose AFM1 exposure. While acute toxicity is unlikely at these levels, the genotoxic and immunosuppressive effects of long-term exposure remain a valid concern [4, 6]. Strengthening on-farm aflatoxin management through improved grain drying, biological control agents such as Aflasafe, and routine feed testing offers the most effective upstream intervention [16, 17].

Conclusion

The main contribution of this research is a multi-city, dual-season, dual-milk-type survey of AFM1 in the Nigerian metropolitan milk supply, executed with a validated competitive ELISA protocol and supported by rigorous quality-control measures including certified reference material and spike-recovery verification.

The supporting evidence can be grouped under four themes. First, AFM1 was detected in nearly nine out of every ten samples, confirming that contamination is endemic across both raw and processed milk channels. Second, raw milk carried significantly higher concentrations than pasteurized milk, with the gap widest during the wet season, pointing to feed-storage conditions as the root cause. Third, geographic variation was significant: Lagos Mainland and Port Harcourt showed the highest levels, likely reflecting the humid coastal climate and the larger informal dairy sector in those cities. Fourth, while virtually all samples complied with the Codex limit of 0.5 µg/L, nearly two-thirds exceeded the EU threshold of 0.05 µg/L, underscoring the regulatory gap that Nigerian consumers face.

The significance of these findings resonates at several levels. For the Nigerian dairy industry, the data make a strong case for investing in pre-harvest and post-harvest aflatoxin mitigation along the feed-to-milk chain. For regulators, the

absence of an enforceable national AFM1 limit specific to liquid milk represents a gap that leaves consumers, especially children, exposed to chronic low-dose carcinogen intake. For the scientific community, the research contributes city-level resolution data that can inform risk-assessment models and exposure calculations tailored to Nigerian dietary patterns. Several questions remain open and deserve targeted follow-up. The carry-over rate from feed to milk in Nigerian dairy systems has not been measured directly; such data would allow back-calculation of the feed-contamination levels required to push milk AFM1 above the EU limit. Whether the same seasonal and geographic patterns apply to fermented dairy products *nono* and *wara*, which are widely consumed in Nigeria is unknown. And the cost-effectiveness of available interventions (Aflasafe biocontrol, hermetic grain storage, activated-clay binders in feed) has not been evaluated under Nigerian smallholder conditions. Answering these questions would move the conversation from surveillance to actionable mitigation.

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Contributions Not Qualifying for Authorship

Mr. Olusegun Adeyemi coordinated sample collection across the six cities. Ms. Blessing Eze assisted with ELISA plate preparation and absorbance readings.

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