

AFLATOXIN CONCENTRATIONS IN MILK FROM HIGH-PRODUCING US HOLSTEINS FED NATURALLY-INFECTED MAIZE AND MILKED 3X PER DAY

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INTRODUCTION

Aflatoxin belongs to a group of fungal toxins known as mycotoxins; these are highly oxygenated, heterocyclic, difuranocoumarin compounds produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Diaz, Calabrese, & Blain, 2008). Aflatoxins are hepatotoxic and carcinogenic secondary metabolic products from these fungal species. Once aflatoxins are produced by the fungi, they are heat, cold, and light stable. They persist to some extent in food even after the inactivation of the fungi by food processing methods, such as ultra-high temperature products, due to their significant chemical stability. Aflatoxins are colorless, odorless, and tasteless. Because even low concentrations can be important, and with the uneven distribution in commodities, aflatoxins are difficult to detect accurately (Peraica, Radić, Lucić, & Pavlović, 1999).

Although *Aspergillus flavus* and *A. parasiticus* grow well in tropical and subtropical climates, they can also be found and produce aflatoxins in more temperate areas (Binder, Tan, Chin, Handl, & Richard, 2007). While fungi are a normal part of the microflora of standing crops and stored feeds, the production of the secondary metabolites, such as aflatoxin B1, are promoted by certain physical and biological factors at points during harvesting, handling, and storage (W. L. Bryden, 2007). Physical factors include moisture, humidity, temperature and mechanical damage of the crops. Biological factors include plant variety, stress from pre-harvest drought, insect damage and spore load.

HEALTH EFFECTS

More than 20 aflatoxin-like secondary metabolites have been identified. Aflatoxin B1 (AFB1) was shown to possess the most toxic and carcinogenic properties to humans and animals (Binder et al., 2007). Along with carcinogenic properties, AFB1 can reduce feed consumption and reduce milk yield in the short term and result in chronic immune suppression and reduced reproductive performance. These chronic effects may be of more economic importance than the acute effects (Bodine and Mertens, 1983). In both humans and animals, AFB1 is metabolized by the liver, creating a hydroxylated metabolite called aflatoxin M1 (AFM1). Both AFB1 and AFM1 are considered group 1a carcinogens (IARC 2007). Aflatoxin M1 is of particular importance to the dairy industry as it is the major aflatoxin metabolite present in mammalian milk.

REGULATIONS

There is current legislation in almost 100 countries regulating the maximum allowable AFB1, total aflatoxin, and AFM1 levels in human food, animal feeds and milk (Berg, 2003). In the US, the FDA creates and enforces action levels for AFB1 and AFM1 in feed, food and milk. These regulatory levels take into account the advisory regulations put forth by the Joint FAO/WHO Expert Committee of Food Additives (JEFCA). In the EU, the European Commission takes into account the advice of the European Food Safety Authority (EFSA) on regulatory levels for AFB1 and AFM1 in feed, food and milk. Both advisory committees work with the Codex Alimentarius Commission of the World Trade Organization to develop harmonized international food standards, guidelines, and codes of practice. The FDA dictates a maximum allowable concentration of 20 µg/kg total aflatoxin in food and feed intended for dairy consumption, and 0.5 µg/kg AFM1 in milk and milk products. These regulations are based mainly on risk analysis of aflatoxins. The European Commission dictates a maximum allowable concentration of 4 µg/kg total aflatoxin in food and feed intended for dairy consumption, and 0.05 µg/kg AFM1 in milk and milk products. These regulations are based on 7 years of occurrence data, risk analysis, and dose-response modeling from animal and epidemiological data. Regulations specific to AFM1 contamination have also influenced regulatory limits in feed for dairy animals. In order to ensure compliance with the maximum levels in milk, stringent maximum levels in dairy feedstuffs are also necessary (European Food Safety, 2004).

AFLATOXIN M1 AND MODERN DAIRY PRODUCTION

A factor that is considered to be important for influencing regulatory limits of both total aflatoxin and AFM1 is the rate at which AFB1 is converted and excreted as AFM1 into the milk of dairy cows (Masoero, Gallo, Moschini, Piva, & Diaz, 2007). The ability of cattle to transform AFB1 in the feed to AFM1 in the milk has been examined in many studies, which demonstrated that such carry-over in dairy cows milked 2 times daily was usually 1% to 2% of the ingested AFB1 for low-yielding cows (< 30 kg milk yield/day) and up to ~6% for high-yielding cows (> 30 kg milk yield/day). Most previous studies on the carry-over of aflatoxins from feed to milk were in what would be considered today as low-yielding dairy cows (Britzi et al., 2013).

Due to specialized breeding programs, technological innovations and other structural changes in milk production in the US, total milk production increased by 45% between 1975 and 2000. There has also been a huge shift from pasture-based dairy operations to confinement feeding operations which has increased the proportion of concentrates such as cornmeal in the feed, and the higher milk yield has increased the overall consumption of feed as well (Blayney, 2002). In order to ensure continued adherence to regulations for AFM1 concentrations in milk, it is now necessary to re-examine the actual transfer of toxicity as aflatoxin in the feed to AFM1 in the milk, and suggest new safety thresholds for feed aflatoxins.

PRELIMINARY WORK

Local survey

We surveyed 38 local farms in upstate NY, taking feed and milk samples to establish a general occurrence level of mycotoxins and formulate a field data carry-over rate for aflatoxin from feed to AFM1 in milk. When possible, we took small samples of TMR from all the bunkers from which the animals on each diet were fed. We then collected milk samples from groups or individual cows from each of the feeding groups. It was often logistically difficult to obtain a good milk sample from just one milking group and on farms without TMR were more difficult to sample cows' actual intakes. It is always difficult to get a representative sample for mycotoxins in feeds because of their heterogeneous distribution (Wayne L. Bryden, 2012). Also, according to other studies, because of the quick excretion rate of aflatoxins in milk, it is hard to collect exactly what feed became part of which milk sample (Decastelli et al., 2007). While we expected farms with unregulated homegrown feed sources to have high milk mycotoxin levels, that was not the case. However we did find that over 14% of the milk samples collected would violate EU regulations for AFM1. We put together a field carry-over graph using 120 points of aflatoxin and AFM1 measurement data (Figure 1). While there was an apparent relationship between feed and milk aflatoxin levels, the specific feed sampled in the bunkers and troughs were assumed to be similar to the feed the cows had consumed to make the milk sampled, but had it usually been fed more recently.

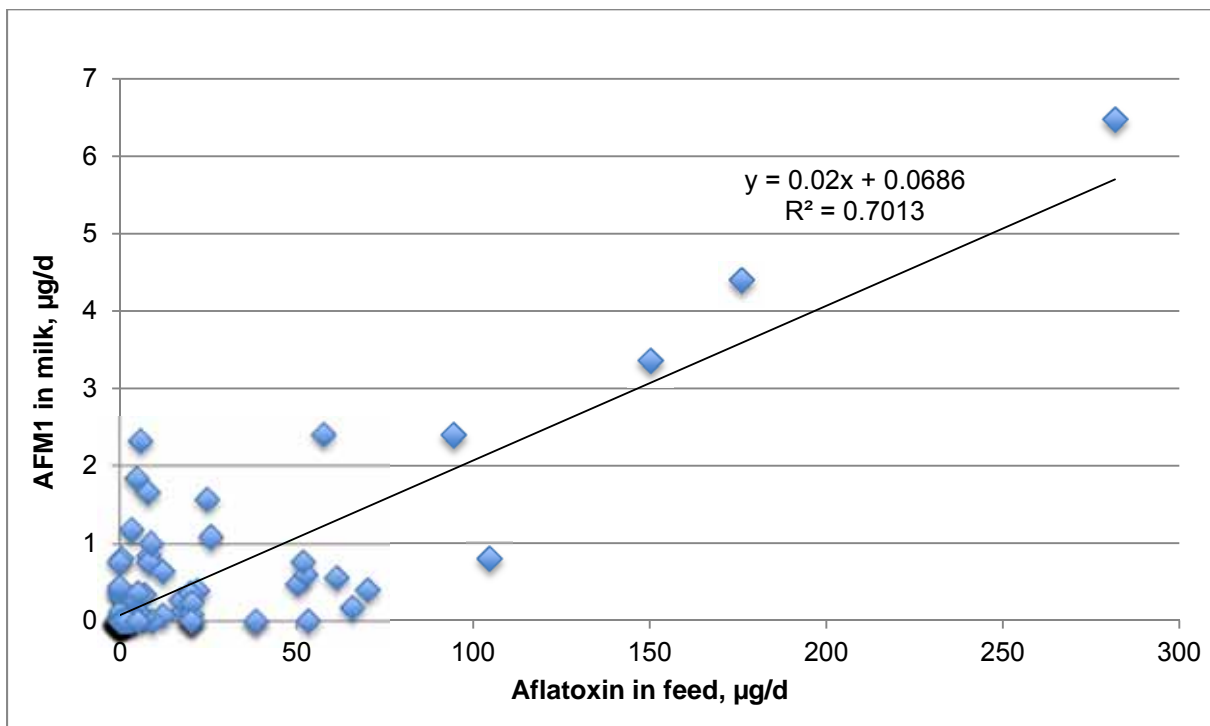


Figure 1. Local field survey data for mass of aflatoxin M1 in milk vs mass of aflatoxin in feed.

Meta-analysis for previous results

We collected individual aflatoxin data and specific methods information from 13 studies from 1967 to 2014 (Figure 2). Some aflatoxin intake data were inferred from methods and other information provided in the papers. Consolidation of these historical works provides an overall picture based on the limited number and scope of past studies. These data are presented together with our field carry-over data in Figure 2 with trend lines added to show approximate carry-over percentages.

Many of the studies represented in Figure 2 used cows with relatively low milk production. The apparent carry-over percentage of 1.2% for these studies is in line with the understanding that lower-producing cows excrete less AFM1 into the milk as a percentage of AFB1 consumed. The relatively low r^2 value for these previous studies may be due to a lack of individual cow data available within each study.

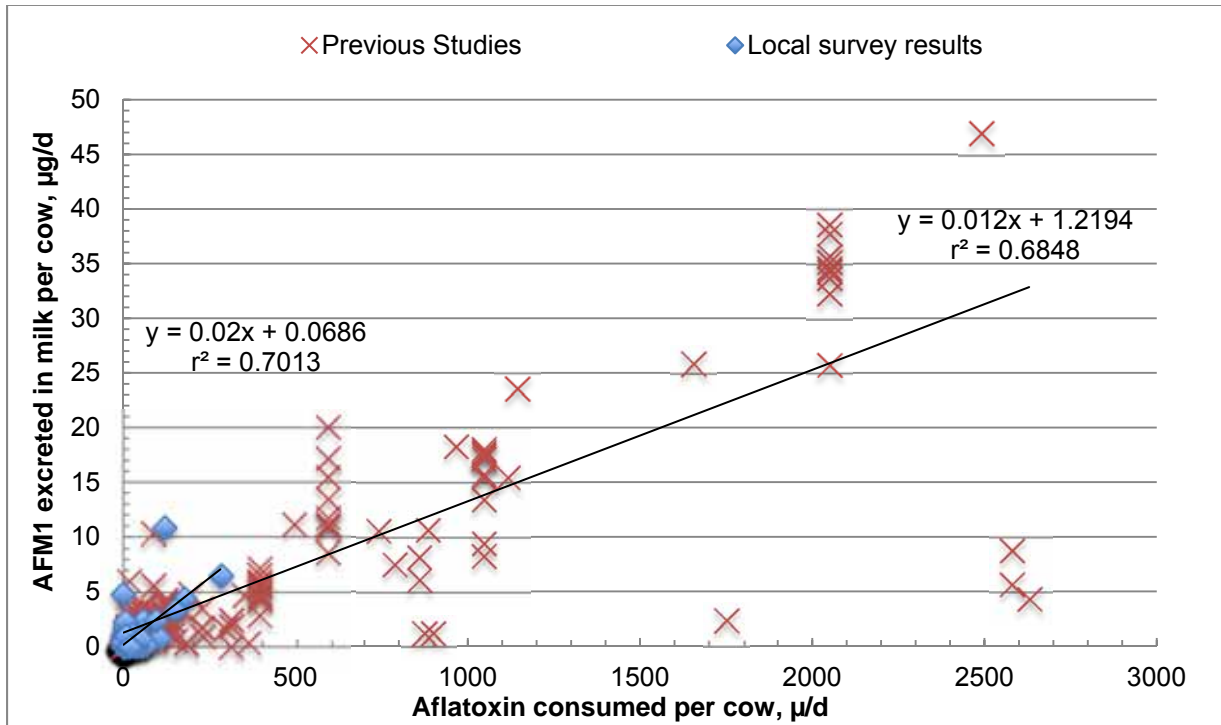


Figure 2. Average mass of AFM1 excreted in milk vs average mass of aflatoxin consumed from field survey data and data collected from previous carry-over experiments (Allcroft, Roberts, & Lloyd, 1968; Applebaum, Brackett, Wiseman, & Marth, 1982; Britzi et al., 2013; Chopra et al., 1999; Frobish, Bradley, Wagner, Long-Bradley, & Hairston, 1986; Lafont, Lafont, Mousset, & Frayssinet, 1980; Masoero et al., 2007; Munksgaard, Larsen, Werner, Andersen, & Viuf, 1987; Patterson, Glancy, & Roberts, 1980; Price, Paulson, Lough, Gingg, & Kurtz, 1985; Veldman, Meijs, Borggreve, & Heeres-van der Tol, 1992).

CONTROLLED FEEDING STUDY

Regulations for maximum limits for aflatoxins in human and animal foods are influenced by experiments to calculate the carry-over of aflatoxins into dairy milk and, in the US, the limit of detection for aflatoxins from 1969 (Binder et al., 2007). In Europe, the expected carry-over rate for aflatoxins into milk is used as part of their worst case scenario calculations for establishing safe maximum limits for aflatoxins in food and milk (Zain, 2011). It is believed that chronic, low level exposure of aflatoxins on high-producing dairy cows will provide a better picture for the current dairy industry and current regulation and safety recommendations (Wayne L. Bryden, 2012). These findings could influence scientific research groups responsible for advising committees in charge of food safety regulations.

Our study used cows with a high milk production level for a more realistic picture of high-producing, intensive dairy operations in the US. We included cows with milk production levels reported as high in only one previous study on mycotoxins (Britzi et al., 2013). While that other study used feed spiked with pure AFB₁, we fed subclinical levels of total aflatoxin through the use of naturally contaminated cornmeal imported from the southeastern US.

Methods

We did three rounds of trials each using 12 high-producing dairy cows in early- to mid-lactation, feeding them naturally contaminated corn meal top-dressed on their daily TMR. One trial lasted 2.5 weeks with a week-long adjustment period to the stalls and the clean TMR, 2 days of infected cornmeal feeding, and milk sampling at all three daily milkings done before, during and after aflatoxin exposure until AFM₁ levels in milk returned to 0 µg/kg. The other two trials were similar but infected cornmeal was fed for 7 days and milk was sampled every other day at all three milkings until AFM₁ levels in the milk returned to 0 µg/kg.

Each trial had 4 cows each fed 1 of 3 diets with: 1) control (0 µg/d), 2) low (300 µg/d), or 3) high (600 µg/d) levels of aflatoxin in the cornmeal. Refusals of the previous day's TMR for each cow were sampled before removing them from the feed buckets. At feeding time each morning, after taking a representative sample of the fresh TMR offered, 1 kg of contaminated cornmeal was top-dressed on the fresh feed for each cow. Samples from each cornmeal bag were collected for each day. Feed samples were taken, refrigerated, and tested in our lab using Aflatest columns and the related published procedure on page 30 of the Aflatest manual from VICAM. Milk samples were taken, refrigerated, and immediately tested in our lab using Afla M₁ FI⁺ columns and the related published procedure on page 11 of the Afla M₁ FI⁺ manual from VICAM. Ingested aflatoxin levels were calculated by adding the aflatoxin in the cornmeal top-dress with the aflatoxin (if any) found in the base TMR and then subtracting any aflatoxin found in the refusals for each cow. Concentrations (µg/kg) of aflatoxin in milk and feed samples were converted to µg of aflatoxin using measured milk yield and DMI.

Results and discussion

Average levels of aflatoxin ingested for each feeding group in each trial are presented in Table 1 as well as average peak levels of AFM1 recorded in the milk for each group in each trial.

Table 1. Average dietary aflatoxin concentration for each feeding group for each trial and average maximum excretion concentration of AFM1 in milk for each feeding group for each trial. Numbers in italics violate the US action levels for aflatoxin and AFM1.

Diet	Trial 1 (7d)	Trial 2 (7d)	Trial 3 (2d)
Aflatoxin ingested ($\mu\text{g}/\text{kg}$ feed DM)			
Control	0	0	0
Low	5.2	12.3	9.4
High	21.7	21.9	16.0
AFM1 excreted ($\mu\text{g}/\text{kg}$ milk)			
Control	0	0	0
Low	0.278	<i>0.543</i>	0.174
High	<i>1.010</i>	<i>0.966</i>	<i>0.504</i>

Total aflatoxin ingested and total AFM1 excreted over the trial period were calculated using measured AFM1 levels in the milk, measured aflatoxin levels in ingested feed, DMI and milk yield at each milking. Using the total aflatoxin ingested and the total AFM1 excreted instead of a daily average of each provides a better overall picture of the carry-over effect without omitting or averaging data during the time to steady-state conditions and the time to 0 $\mu\text{g}/\text{kg}$ AFM1 in the milk following the cessation of dosing. Linear regression was used to calculate the direct carry-over into milk as 6.5 $\mu\text{g}/100 \mu\text{g}$ consumed (Figure 3).

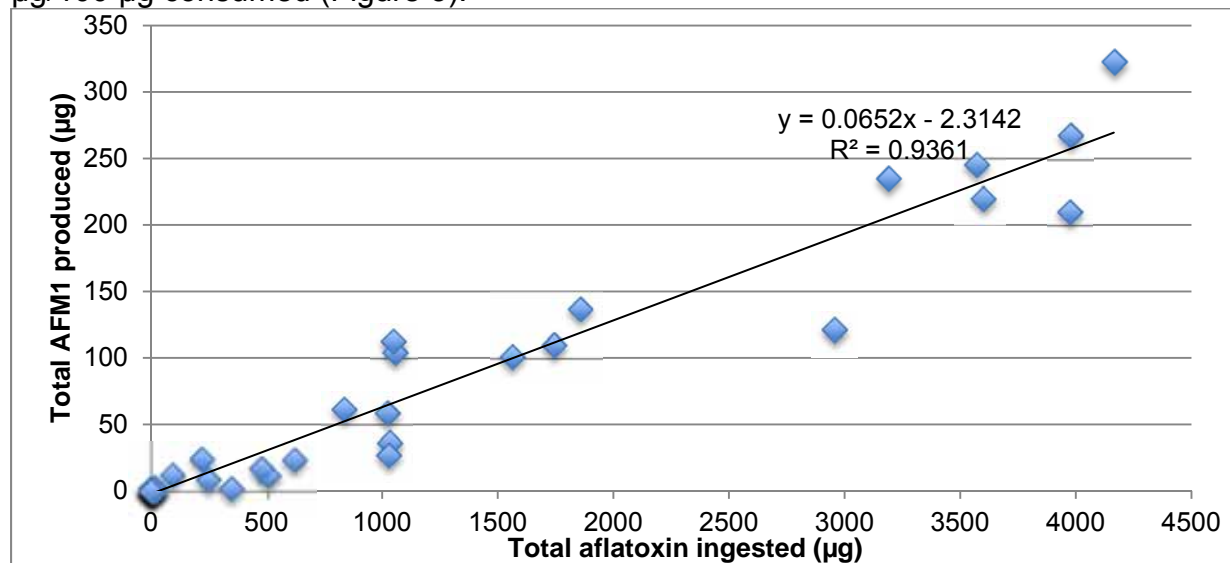


Figure 3. Individual cow data for total mass of AFM1 excreted in milk vs mass of aflatoxin ingested from the feed over the trial period.

Average concentrations of ingested aflatoxin in feed and average concentrations of excreted AFM1 in milk were calculated for each cow in the low and high groups for the experimental feeding period (Figure 4). The vertical and horizontal red lines mark the US regulatory limits for total aflatoxin in feed and aflatoxin M1 in milk respectively. Linear regression was used to calculate the relationship between ingested and excreted concentrations of aflatoxin and AFM1. The linear regression line crosses the line marking the US regulatory limit for AFM1 in milk at an aflatoxin level of 15 $\mu\text{g}/\text{kg}$ (ppb) in the feed suggesting that this level of aflatoxin in the feed is the maximum likely to produce milk below the US regulatory limits.

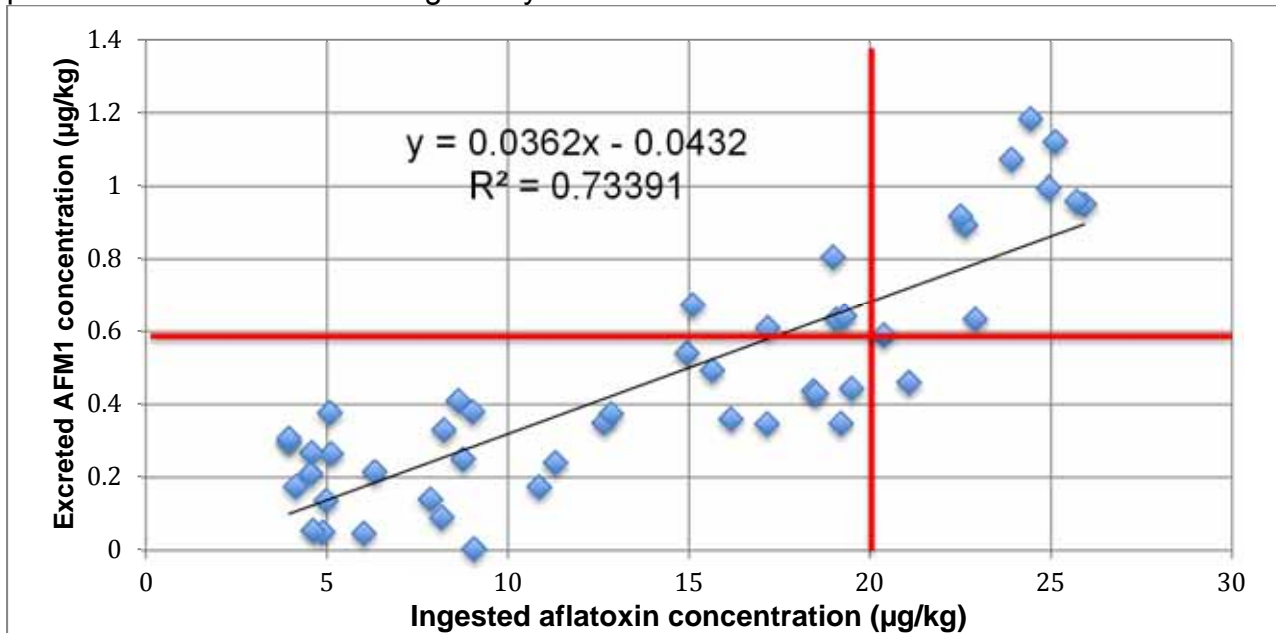


Figure 4. Average concentration ($\mu\text{g}/\text{kg}$) of excreted AFM1 for each cow over the experimental feeding period vs average concentration ($\mu\text{g}/\text{kg}$) of ingested aflatoxin for each cow.

FUTURE IMPLICATIONS AND RECOMMENDATIONS

While commercial feed and some milk production operations screen for a range of mycotoxins, only aflatoxin is subject to action levels by the FDA. In the US, milk is not regularly screened for mycotoxins on a commercial or small farm level. While we did not observe a concerning number of feed or milk samples taken from small dairy farms in upstate NY that violated US regulatory limits, our trial put to the test the safety of feeding high-producing dairy cows feed with the legal limit of aflatoxin present. Our data suggest very strongly that the current “safe” limits of aflatoxin allowable in feed for dairy cows do not protect against violating the current regulations for AFM1 residue in the resulting milk. The carry-over percentage of 6.5% found from our study shows that high-producing dairy cows will have a higher carry-over percentage than the 1 to 2% that has been suggested by previous studies using low-producing cows.

These findings suggest that the current regulations of 20 $\mu\text{g}/\text{kg}$ total aflatoxin levels allowable in dairy cow feed are not protective to avoid violation of the 0.5 $\mu\text{g}/\text{kg}$

AFM1 regulatory levels for milk in high-producing cows. Farmers should be vigilant about proper harvesting, storing, and regular testing of feedstuffs for dairy cows to ensure the safety of the animals and the humans consuming their products.

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