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Analysis of Formulated Cattle-Feed Samples for the Natural Occurrence of Storage Fungi and Aflatoxin B1

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A B S T R A C T

Contamination of feed materials with aflatoxins is an important issue due to both acute and chronic intoxication in animals and also harmful to humans. In the present study, 25 formulated cattle feed samples of different sources were collected from Tamil Nadu and analysed for moisture content, storage mycoflora including aflatoxigenic *A. flavus* and aflatoxin B_1. The results show that the moisture content of the cattle feed samples varied from 7.0% to 14.6% with an average of 10.34%. The storage fungi occurred in the range of 0 to 1,600 cfu/g with an average of 340 cfu/g. Different species of storage fungi occurred in the cattle feed samples are *A. glaucus, A. terreus, A. niger, Penicillium speices, A. flavus, A. fumiatus* and *A. candidus* in the order of dominance. Among the species individual fungi, *A. glaucus* occurred frequently which is a remarkable incidence in the samples collected. Out of 25 samples analysed for the presence of aflatoxin B_1, 20 were positive for aflatoxin B_1 and 5 were negative. The quantity of aflatoxin B_1 in cattle feed samples ranging from 5 ppb to 60 pbb with an average of 20 pbb. Out of 10 A. flavus strains isolated 5 were found produced aflatoxin B_1. It is concluded that from the study as the majority of cattle feed samples analysed from the market is observed to be safe level for the contamination with aflatoxinB_1 and storage fungi.

Introduction

Contamination of feeds with mycotoxins including aflatoxins accounts for significant economic losses in animal husbandry as well as in undesirable trade barriers for raw materials and consumable products (Wu 2006). The Food and Agriculture Organization (FAO**)** estimates that many basic food and feeds material could be contaminated with mycotoxin producing fungi, contributing to huge global losses, about 1000 million metric tons each year (Bhat, Rai, & Karim, 2010). The occurrence of aflatoxins in feed materials also a major problem for farmers due to both acute and chronic intoxication in animals subsequently to human. The impact of feed contamination with mycotoxins includes productivity reduction and organ damage (Upadhaya, Park, & Ha, 2010). The primary source of aflatoxins in cattle feed is due to the contamination of aflatoxin producing *Aspergillus flavus* in agricultural commodities including cereal grains and oilseeds such as rice, corn, peanuts, cottonseed, millet, sorghum and other feed grains which are main ingredients used for formulation of cattle and

poultry. Aflatoxin is the most problematic in dairy due to its derivative aflatoxin M1 (AFM1) present in milk, and its potential health hazard for human consumption. The studies of contamination of aflatoxins in agricultural commodities like maize, rice, wheat and other crops including oilseeds like peanuts have been reported in many parts of India from various research Institutes (Oranusi and Olarewaju, 2013; Vasanthi *et al.,* 2012; European Union, 2011; Reddy *et al.,* 2008; Toteja et al, 2006; Bilgrami *et al.,*1980). The natural occurrence of aflatoxins in various feed materials were reported by Whitlow *et al.,* (2016), Korrapati *et al.,* (2015), Kangetha and Langa (2009), Johanna (2008) which indicate the contamination of aflatoxins is threat to health.

Mycotoxins are ubiquitous in agricultural commodities and produced by several fungi, particularly manyspecies of *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps* and *Alternaria* etc. Natural occurrence of aflatoxins in raw agricultural products poses severe health and economic risks worldwide. The remarkable contamination of aflatoxin B_1 in rice bran, de-oiled bran, Jatropha seeds, cattle-feed and M_1 in milk were analysed in our earlier studies, which indicate health threatening in humans as well as animals (Jayaraman *et al.,* 2011; Jayaraman and Kalyanasundarm, 2009; Jayaraman and Kalyanasundaram, 1994; Jayaraman, 1991; Jayaraman and Kalyanasudaram, 1990). The studies shows that among the species of mycotoxins producing fungi, *Aspergillus flavus* sub sp. *parasiticus* is a potent aflatoxin producing fungi occurred frequently in stored food grains, rice bran as well as in the freshly harvested grains before storage were reported (Jayaraman and Kalyanasundaram, 1994; Jayaraman and Kalyanasundaram, 1992; Jayaraman and Kalyanasundaram, 1990; Bhanumathy and Kalyanasundaram, 1986, Gajapathy and Kalyanasundaram, 1986; Bilgrami *et al.,* 1980;). The natural occurrence of aflatoxins in rice bran and its products indicates that the formulated cattle feed with rice bran may possible for the contamination with aflatoxin. The remarkable contamination of aflatoxin B_1 in rice bran, de-oiled bran and cattle-feed and M_1 in milk were reported in our earlier studies (Jayaraman and Kalyanasundarm, 2009; Jayaraman and Kalyanasundaram, 1994), which indicate health threatening in humans as well as animals.

Based on the above study, the investigations, the present study is made for survey on contamination of aflatoxin B_1 and storage fungi including toxigenic *Aspergillus flavus* in cattle-feed samples collected from different sources like local markets, and dairy farms of Tamil Nadu.

Materials and Methods

Samples Collection

The formulated cattle feed samples were collected from the markets, manufacturers and dairy farm from Chennai, Chengalpet, Kanchipuram, Maduranthagam, Vellore and Thiruvannamalai districts of Tamil Nadu. The samples were collected in 500 g quantity by random sampling method in preclened plythene bags and closed tightly with rubber brands. Then the samples were labelled properly for source, date of collection and place of collection and kept in plastic container in the laboratory for further analysis.

Determination of Moisture Content

The moisture content of the composite cattle feed samples were determined by hot-air oven drying method at 100^{o}C temperature for 1 hour period. Exactly 5 g of cattle feed sample

was taken for drying in oven and the loss of weight was calculated. The method was standardised till obtained same value of loss of weight by repeated drying. The percentage of moisture content of cattle feed samples were calculated and expressed on wet weight basis.

Analysis of storage fungi

The storage mycoflora of the cattle feed samples were analysed by using serial dilution plate technique. One ml aliquots of the serially diluted samples were plated aseptically over the agar medium and the plates were swirled to distribute the inoculum uniformly entire surface area. To enumerate the storage fungi which are usually grow after storage on agricultural raw materials for cattle feed formulation, a highly osmotic selective medium called Czapek's Dox Agar (CDA) (Rao & Kalyanasundaram, 1983) containing 50% sucrose w/v sucrose was used throughout the study. The media included antibiotic streptomycin and penicillin in mentioned concentration to suppress any external contamination. Then the inoculated plates were incubated at 30 +/- 1 ^{o}C up to one week for development of fungal colonies. The individual species of storage fungi were observed separately and their numbers were expressed as colony forming units per gram (cfu/g) of cattle feed samples. The different species of *Aspergillus* and *Penicillium* were identified according to standard methods used (Jayaraman and Kalyanasundaram, 1994). The individual fungal species were also maintained on agar slants of normal Czapek's Dox Agar with 3% sucrose for further work.

Aflatoxin Analysis

TLC method

The extraction, isolation and assay of aflatoxins from cattle feed samples were done by the simple screening method by Seitz and Mohr (1972) using methanol as a solvent and separation by Thin Layer Chromatrography. The sample spotted TLC plates were developed in chloroform : acetone (88:12) as a developing solvent. The identification of aflatoxin B_1 was done using fluorescent UV lamp at short wave length (354 nm). The presence of blue fluorescence is the indication of positive result. The samples were run along with standard aflatoxin B_1 for comparison. The quantitative estimation of the aflatoxin from samples was also made by the spectrophotometric method described by Nabney & Nesbitt (1965). Aflatoxin recovered in cold methanol from silica gel plate was read spectrophotometerically at 363 nm and 420 nm and the OD values were taken for calculation. The following formula was used for quantitative estimation.

ELISA method

The in-house standard method of ELISA for determination of aflatoxin B_1 developed by ICRISAT, Hyderabad was used for further confirmation. The composite cattle feed samples were taken for extraction of aflatoxin by using solvents and the condensed samples containing aflatoxin if any is stored in small glass vials. Aflatoxin B_1-BSA conjugate and aflatoxin B_1 antibodies were used for Enzyme Linked Immunoadsorbent assay of aflatoxins from samples. The micro-well ELISA plates containing 96 wells were used for reactions and the OD values were optically measured by microplate reader at 450 nm. The OD values of the samples were compared with standard smples (aflatoxin B_1 -BSA conjugate) and determined the results of samples.

Test for Toxigenicity of *Aspergillus flavus*

To screen the fungal isolates for production of aflatoxin in vitro, the fungi were cultured in

slants of an agar medium containing 2% yeast extract and 15% sucrose (YES). Toxins were extracted from the molten agar with chloroform and assayed by TLC using toluene: ethyl acetate: 90% formic acid (6:3:1) solvent system (Bullerman, 1974). The fluorescent spots in blue and green TLC under short wavelength indicates the presence or absence of aflatoxin and the toxigenic property of the *Aspergillus flavus* strains.

Results and Discussion

Samples

Totally 25 cattle feed samples were collected from different sources in markets and cattle farms of Kancheepuram, Thiruvallur, Villupuram, Thiruvannamalai and Vellore districts of Tamil Nadu. The cattle feed samples collected for the present study were belong to 8 different market brands available in dealers and cattle farms from respective manufacturers. All the samples were appeared in the form of compressed and dried pellets in various lengths of 2 cm to 5 cm with 0.5 cm breath. The details of samples were as Brand 1 (8 samples – Sample 1, 4, 10, 14, 19), Brand 2 (10 samples – Sample No. 3, 5, 7, 8, 11, 12, 13, 18, 23, 24), Brand 3 (1 sample - sample 2), Brand 4 (1 sample - sample 15), Brand 5 (1 sample - sample 16), Brand 6 (2 samples – 6, 17), Brand 7 (3 samples – sample 21, 22, 25) and Brand 8 (2 samples – sample 9, 20). The details of sample collection is presented in Table 1.

Moisture content in cattle feed samples

The moisture content of the cattle feed samples varied from 7.0% to 14.6% with an average of 10.34%. The lowest moisture content percentage was observed in samples 2, 5, 9 and 15 as 7.0% and the highest moisture content was observed in samples 12 and 13 as 14.6%. The details of moisture content from individual samples were presented in Table 1 and Fig. 1.

Storage fungi

The storage fungi mainly comprising different species of *Aspergillus* and *Pencillium* were encountered in cattle feed samples in varying numbers. Few other fungi like *Mucor, Rhizophus, Pyricularia, Helminthosporium and cladosporium* also were observed in very less numbers in cattle feed sampes. The following individual species of *Aspergillus* and *Penicillium* were observed.

1. *Aspergillus niger*
2. *A. glaucus*
3. *A. flavus*
4. *A. terreus*
5. *A. nidulans*
6. *A. fumigates*
7. *A. candidus*
8. *Penicillium citrinum*
9. *P. Funiculosum*
10. *P. Chrysogenum*
11. *P. tardum*
12. *Mucor mucedo*
13. *Rhizophus stolonifer*
14. *Pyricularia oryzae*

The overall population of storage fungi in individual cattle feed samples is observed as in the range of 600 to 1600 cfu/g with an average of 950 cfu/g. Among the above species of fungi, *Aspergillus glaucus* observed very frequently in samples and dominance in samples in the range of 0 to 1,600 cfu/g with an average of 340 cfu/g. followed by *A. terreus, Penicillium citrinum and A. fumigatus* occurred in less numbers. However, the overall population of storage fungi is observed to be very less when compared with the population of various rice bran samples in our earlier studies which are one of the raw material for formulation of cattle feed. Our earlier studies shows the occurrence of storage fungi in rice bran and de-oiled rice bran in higher numbers as up to

1,30,000 cfu/g with an average of 33,000 cfu/g which include toxigenic A. flavus (Jayaraman and Kalyanasundaram, 2009; Jayaraman, 1991). The details were presented in Table 2 and Fig. 2.

Aflatoxins

Out of 25 cattle feed samples analysed, 20 samples were found positive results for aflatoxin B_1. Five samples were found free of contamination with aflatoxin B_1. The concentration of aflatoxin B_1 in different cattle feed samples varying in the range from 5 ppb to 110 ppb with an average of 22 ppb. Of the 25 samples from 8 brands of cattle feed analysed, the samples 4, 8, 9, 15 and 24 showed negative for aflatoxin B_1.

Table.1 Details of moisture content (%) of cattle feed samples

S. No.	Name of the sample	Place of Collection	District Name	Moisture Content (%)
1	Sample 1	MM Nagar	Kancheepuram	9.6
2	Sample 2	Chengalpet	Kancheepuram	7.0
3	sample 3	Maduranthagam	Kancheepuram	8.8
4	Sample 4	Chengalpet	Kancheepuram	10.8
5	Sample 5	Uthiramerur	Kancheepuram	7.0
6	Sample 6	Thiruvallur	Thiruvallur	9.4
7	Sample 7	Palamarathur	Thiruvannamala	8.2
8	Sample 8	Palamarathur	Thiruvannamala	9.0
9	Sample 9	Kattangulathur	Kancheepuram	7.0
10	Sample 10	MM Nagar	Kancheepuram	8.6
11	Sample 11	Uthiramerur	Kancheepuram	12.4
12	Sample 12	Uthiramerur	Kancheepuram	14.6
13	Sample 13	Chetpet	Thiruvannamalai	14.6
14	Sample 14	Manalurpet	Villupuram	13.2
15	Sample 15	Gingee	Villupuram	7.0
16	Sample 16	Gingee	Villupram	12.2
17	Sample 17	Chetpet	Thiruvannamalai	10.4
18	Sample 18	Chetpet	Thiruvannamalai	13.2
19	Sample 19	Chengalpet	Kancheepuram	8.6
20	Sample 20	Kattankolathur	Kancheepuram	12.6
21	Sample 21	Madhuranthagam	Kancheepuram	10.4
22	Sample 22	Madhuranthagam	Kancheepuram	10.6
23	Sample 23	Palamarathur	Thiruvannamalai	11.6
24	Sample 24	Palamarathur	Thiruvannamalai	12.8
25	Sample 25	Palamarathur	Thiruvannamalai	8.8

Table.2 Occurrence of storage fungi and toxigenic fungi in cattle feed samples

S. No.	Name of the sample	Place of Collection	Total No. of Storage fungi (CFU/g)	*A. flavus*	Afla-toxigenic *A.flavus*	*A. glacus*
1	Sample 1	MM Nagar	1600	400	+	1000
2	Sample 2	Chengalpet	1200	0	-	500
3	sample 3	Maduranthagam	600	200	-	0
4	Sample 4	Chengalpet	800	0	-	200
5	Sample 5	Uthiramerur	1000	0	-	500
6	Sample 6	Thiruvallur	1200	0	-	600
7	Sample 7	Palamarathur	900	0	-	0
8	Sample 8	Palamarathur	800	0	-	200
9	Sample 9	Kattangulathur	1100	900	+	0
10	Sample 10	MM Nagar	700	200	-	0
11	Sample 11	Uthiramerur	1000	0	-	0
12	Sample 12	Uthiramerur	900	200	-	200
13	Sample 13	Chetpet	500	200	+	100
14	Sample 14	Manalurpet	1000	200	-	0
15	Sample 15	Gingee	1200	0	-	200
16	Sample 16	Gingee	1600	0	-	1000
17	Sample 17	Chetpet	1000	300	+	600
18	Sample 18	Chetpet	600	200	+	300
19	Sample 19	Chengalpet	1600	0	-	1600
20	Sample 20	Kattankolathur	800	0	-	600
21	Sample 21	Madhuranthagam	500	0	-	500
22	Sample 22	Madhuranthagam	600	0	-	500
23	Sample 23	Palamarathur	600	0	-	0
24	Sample 24	Palamarathur	800	0	-	0
25	Sample 25	Palamarathur	1100	0	-	400

Table.3 Occurrence of aflatoxin B1 in different cattle feed samples

S. No.	Name of the sample	Place of Collection	Aflatoxin analysis	
			Qualitative	Quantitative (PPb)
1	Sample 1	MM Nagar	+	5
2	Sample 2	Chengalpet	+	5
3	Sample 3	Maduranthagam	+	5
4	Sample 4	Chengalpet	-	Not detected
5	Sample 5	Uthiramerur	+	10
6	Sample 6	Thiruvallur	+	25
7	Sample 7	Palamarathur	+	10
8	Sample 8	Palamarathur	-	Not detected
9	Sample 9	Kattangulathur	-	Not detected
10	Sample 10	MM Nagar	+	10
11	Sample 11	Uthiramerur	+	15
12	Sample 12	Uthiramerur	+	15
13	Sample 13	Chetpet	+	20
14	Sample 14	Manalurpet	+	5
15	Sample 15	Gingee	-	Not detected
16	Sample 16	Gingee	+	50
17	Sample 17	Chetpet	+	15
18	Sample 18	Chetpet	+	5
19	Sample 19	Chengalpet	+	60
20	Sample 20	Kattankolathur	+	10
21	Sample 21	Madhuranthagam	+	110
22	Sample 22	Madhuranthagam	+	50
23	Sample 23	Palamarathur	+	10
24	Sample 24	Palamarathur	-	Not detected
25	Sample 25	Palamarathur	+	5

+ Positive result; - Negative result

Fig.1 Details of moisture content (%) of cattle feed samples

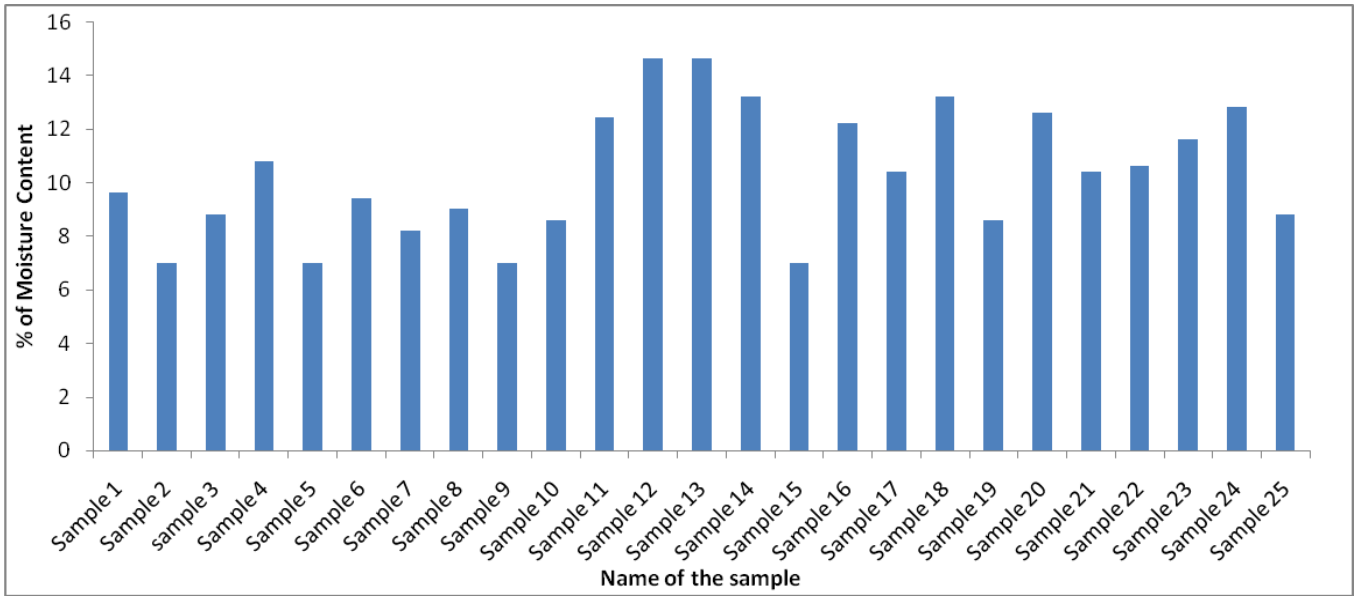
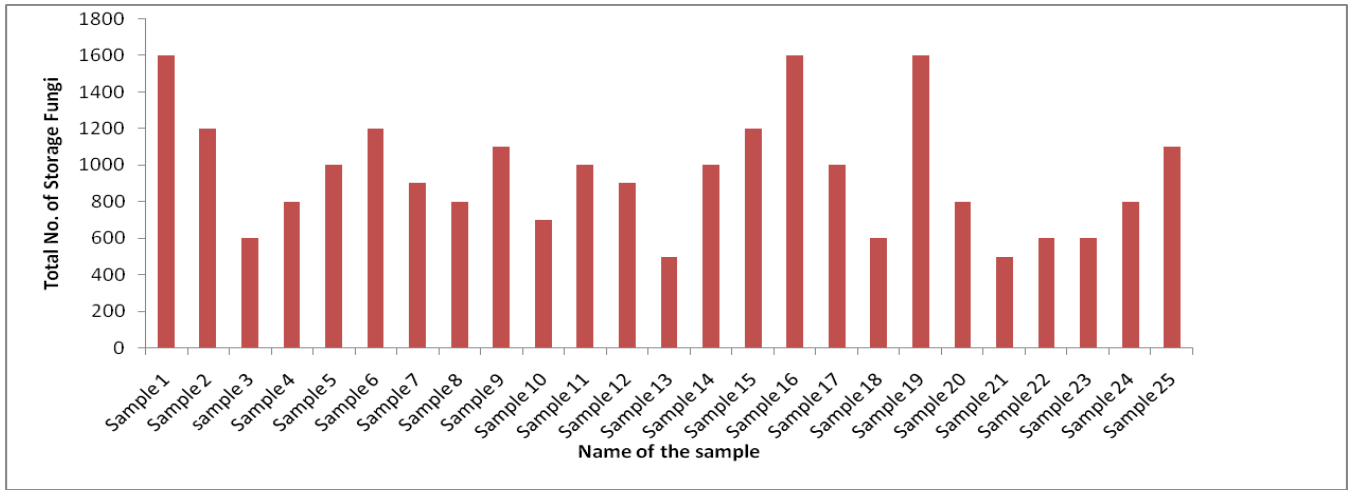


Fig.2 Occurrence of storage fungi and toxigenic fungi in cattle feed samples



Among the samples found positive for aflatoxins, samples 16, 19, 21 and 22 showed slightly higher concentration (50 ppm, 60 ppm, 110 ppm and 50 ppm respectively) and other samples show very lower concentration. These differences might be due to the presence aflatoxin contamination in raw materials used for formulation of cattle feed and the processing parameters. Contamination of deoiled rice bran and carttle feed samples with higher level of aflatoxins in our earlier studies (Jayaraman and Kalyanasundaram, 2009,; Jayaraman and Kalyanasundaram 1994, Jayaraman, 1991) is not comparable to the cattle feed samples of the present study which might be due to the advancement of processing technology in cattlefeed manufacturing as well as raw material quality. However, the lower concentration or absence of aflatoxin B_1 in cattle feed samples will yield very lower level of occurrence in milk samples due to the metabolic degradation of aflatoxins in dairy animals. The occurrence of distribution of aflatoxin B_1 in cattle feed samples were presented in Table 3.

Toxigenic fungi

Out of 25 cattle feed samples analysed for the storage fungi, only 9 samples were found contaminated with of *Aspergillus flavus* a lower population as 100 cfu/g to 300 cfu/g of sample (sample 1, sample 3, sample 9, sample 10, 12, 13, 14, 17 and 18). Out of 9 strains of *A. flavus*, only 4 strains (sample 1, sample 9, sample 13, sample 17 and 18 respectively) were found to produce aflatoxins B_1 (Table 3). This indicates that the cattle feed samples show mostly free of contamination with *A. flavus* species. However in the previous studies of Jayaraman and Kalyanasundaram, 2009 and Jayaraman and Kalyanasundaram, 1990, more than 60% of *A. flavus* strains isolated from rice bran and de-oiled bran samples were positive for aflatoxin production. The lower occurrence and less toxigenci *A. flvus t*his might be due to the physical and chemical parameters involve in the treatments during processing of manufacture.

In conclusion, the present study revealed that the presence of aflatoxin B_1 in most of the cattle feed samples available in the compounded form at lower concentration as safer level except very few samples. The population of storage fungi including *Aspergillus and Pencillium* species also was observed to be very low when compared with raw materials used for formulation before processing. The remarkable finding of the present study is thelowe incidence of aflatoxin producing *Aspergillus flavus* in cattle feed samples which indicate the further contamination is seldom possible even during storage of cattle feed. Based on the above results it is found most of the cattle feed samples available in the market is contaminated with permissible limit of aflatoxin B_1 which even possible to reduce further after deriving in to milk durig metabolic degradation in cow. However, survey work of the above is undergoing and the final conclusion could be obtained after making large number of samples by using ELISA technique.

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