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MYCOTOXIN DETECTION IN INFANT FORMULA MILKS IN ITALY

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ABSTRACT

After birth, infant formulas constitute an important or often sole food source for infants during the first months of life. In this study, a survey on the presence of aflatoxin M₁ (AFM₁) and ochratoxin A (OTA) in the 14 leading brands of infant formulas marketed in Italy was conducted. Mycotoxins were determined by immunoaffinity column clean-up and HPLC with fluorescence detection. AFM₁ was found in 2 out of 185 samples but at levels below the European legislation limit of 25 ng/L. OTA was detected in 133 (72%) samples (range 35.1-689.5 ng/L). It has been observed that OTA contamination was 80% in the ready-to-use preparations and 63% in the powdered samples. The Scientific Committee for Food (SCF) reviewed the toxicology on OTA and concluded that it would be prudent to reduce exposure to OTA ensuring that exposure is towards the lower end of the range of tolerable daily intakes of 1.2-14 ng/kg b.w. per day. OTA was also evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and a provisional tolerable weekly intake (PTWI) of 100 ng/kg b.w. was established. The OTA levels in pre-term ready-to-use infant formulas were sufficient to cause a higher OTA intake than the suggested TDI. Our results point out the need to perform controls for prevention programs especially when attempting to identify risk markers of the infant feed quality.

Key Words: ochratoxin A, aflatoxin M₁, milk, infant formula, Italy, child health.

Word count of abstract: 224

Abbreviations. Aflatoxin M₁ (AFM₁); Aflatoxin B₁ (AFB₁); High Performance Liquid Chromatography (HPLC); Immunoaffinity Columns (IAC); Ochratoxin A (OTA); Tolerable Daily Intake (TDI).

INTRODUCTION

Aflatoxins and ochratoxins are highly toxic secondary metabolites produced by several *Aspergillus* and *Penicillium* spp that can be found in different commodities including cow's milk. Aflatoxin M1 (AFM1), the major metabolite of aflatoxin B1 (AFB1), and ochratoxin A (OTA) are classified by the International Agency of Research on Cancer (IARC) as class 2B, possible human carcinogens (IARC, 1993a; 1993b). AFM1 level in milk and in milk products is regulated in several countries; the European Union (EU) limit of 0.05 µg/kg being one of the lowest in the world (EC, 2003a). In contrast, no regulation for OTA in milk exists, even though it has been suggested that OTA level in cow's milk may exceed the tolerable daily intake (TDI) of 5 ng/kg b.w. per day for small children in some areas in Norway (Skaug, 1999).

The Scientific Committee for Food (SCF) reviewed the toxicology on OTA and concluded in its opinion on OTA in food on the 17th September 1998 (SCF, 1998) that there is an increasing concern about potential genotoxicity of OTA and its mechanism of action as a carcinogen. Therefore the Committee considered it would be prudent to reduce exposure to OTA as much as possible, ensuring that exposures are towards the lower end of the range of tolerable daily intakes of 1.2-14 ng/kg b.w. per day. OTA was also evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and a provisional tolerable weekly intake (PTWI) of 100 ng/kg b.w. was established (FAO/WHO, 2001). The European Food Safety Authority (EFSA) Scientific Panel on Contaminants in the Food Chain in considering all the toxicological and mechanistic data, established a Tolerable Weekly Intake (TWI) of 120 ng/kg body weight (EFSA, 2006).

Aflatoxins are predominantly produced in hot climates. In temperate countries, the strict control of imported feeds has limited the cases of milk contamination above the maximum tolerable limits. However, the presence of aflatoxins was recently reported in feeds

grown in Europe (EC, 2003b; EFSA, 2004). The emergence of aflatoxins in the European continent is concurrent with the increase in the annual average temperature registered in the past decade (Anderson et al., 2004).

Investigations on milk AFM1 contamination are regularly conducted in European countries (EFSA, 2004), but there is little information on the milk contamination by other mycotoxins. In particular, there is scarce information on the presence of OTA in cow's milk (Skaug, 1999; Breitholtz-Emanuelsson et al., 1993; Valenta and Goll, 1996), and no data are available in Italy concerning infant milk-based formula contamination by this mycotoxin. Consumption of cow's milk has not previously been considered as possible source of OTA intake, because of the microflora in the rumen hydrolyses OTA to the less toxic metabolite ochratoxin α (Hult et al., 1976; Sreemannarayana et al., 1988). However, OTA has been found in bovine milk, in low concentrations, indicating that small amounts may escape rumen metabolism and are absorbed (Skaug, 1999; Breitholtz-Emanuelsson et al., 1993). The OTA concentration in cow's milk is expected to be very low, but even low concentrations might markedly increase OTA daily intake by consumers of large quantities of milk such as children, and contribute to significant portion of the total dietary intake (Skaug, 1999).

After birth, infant formulas constitute an important or often sole source of food for newborns and infants during the first months of life. Thus, these formulas have a special role in the infant diet because they should serve as substitutes for human milk. It is desirable that infant formulas should be proportionally similar or inferior in AFM1 and OTA levels to those found by human milk. In general, AFM1 and OTA concentrations in breast milk show marked variability depending on mother's life style and dietary habits. OTA was detected in milk from healthy mothers in Germany, Norway, Sweden, Hungary, Italy, Australia and Sierra Leone (Scott, 2005). Previous data from Italy had indicated a high prevalence of OTA in human milk samples with concentrations ranging between 1700-6600 ng/L and 100-12000

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ng/L, respectively (Micco et al., 1991; 1995). A second study has been carried out in Italy (Turconi et al., 2004). OTA was detected in 198 samples (85.7%) at an average value of 6.01 ng/L. More recently, data from milk samples collected from 82 Italian women indicated that 74% of human milk samples contained OTA in the range < 5 ng/L-405 ng/L (Galvano et al., 2008). In the same study AFM1 was detected in four (5%) of milk samples (ranging from < 7 ng/L to 140 ng/L) (Galvano et al., 2008). Relatively little work on the topic of mycotoxins in infant food has been reported in the literature (Galvano et al., 1998; 2001; Lombaert et al., 2003; Tam et al., 2006; Beretta et al., 2002). Most data have only been part of larger surveys of cereal foods (Lombaert et al., 2003; Tam et al., 2006; Beretta et al., 2002).

For these reasons, the aim of the following work was to determine AFM1 and OTA levels in the leading brands of infant formulas marketed in Italy, and then, to assess their repercussion in the provisional tolerable weekly intake of OTA and of AFM1.

METHODS

Samples

From 14 commercial brands commonly marketed in Italy, a total of 185 samples (5 different batch numbers for each product) of infant formula powders ($n = 17$) and ready-to-use preparations ($n = 20$) were collected from 2006 to 2007 and analyzed. Cow's milk-based formulas were classified as pre-term formula ($n = 11$; 1 powder and 10 liquid) and starter formula for infants from the first day to 6 months of age ($n = 26$, 16 powder and 10 liquid). Table 1 and 2 report their main characteristics.

Standard preparation

OTA (from *Aspergillus ochraceus*) (M 403.8) and AFM1 (from *Aspergillus flavus*) (M 328.2) reference standards, acetic acid (analytical reagent grade) and Celite were purchased from Sigma (Milan, Italy). The OTA standard was dissolved in a toluene-acetic acid mixture (99:1 %, v/v) to give a stock solution of 200 $\mu\text{g/ml}$ which was stored at -20°C until use. Working solutions were prepared by diluting the stock solution with the mobile phase consisting of methanol-sodium phosphate buffer (pH 7.5) 45:55 v/v. The AFM1 standard was dissolved in a acetonitrile to give a stock solution of 10 $\mu\text{g/ml}$ which was stored at -20°C until use. Working solutions were prepared by diluting the stock solution with the mobile phase consisting of acetonitrile-water 23:77 v/v. HPLC-grade water, methanol and acetonitrile were supplied by LABSCAN (Hasselt, Belgium). The immunOTAffinity columns (IAC) AflaStar® and OchraStar® were purchased from Tecna Srl (Trieste, Italy).

Mycotoxins are highly toxic substances, precautions must be observed when handling these substances, safety instructions for working with mycotoxins are given in Valenta (1998). All mycotoxins waste and contaminated laboratory materials were kept for 24 h in sodium hypochlorite solution (household bleach) for decontamination.

Samples preparation

OTA. Five ml of infant formula samples were mixed with 15 ml of extraction solution (water/methanol 50/50 v/v). Extraction was done by shaking for 5 min on horizontal shaker and centrifuging at 3000 rpm for 10 min. The extract (4 ml) was diluted with 46 ml of phosphate-buffered saline (PBS, pH 7.4). The diluted sample was filtered through Whatman filter paper (Millipore Corporation, Maid Stone, UK). This solution (50 ml) was passed through IAC at flow-rate of 1–2 drops s⁻¹. The column was washed with 20 ml of PBS (1–2 drops s⁻¹). Elution was performed with 3 ml of methanol and acetic acid 98/2 v/v. The elute was evaporated to dryness under nitrogen stream. The residue was re-dissolved in 250 µl of HPLC mobile phase and injected into HPLC system. For samples of powdered milk 5 g were suspended in 30 ml of distilled water, heated to 50°C, homogenized and then processed as for liquid milk.

AFM1. Milk samples were previously skimmed by means of addition of Celite and centrifugation at 3000 rpm for 10 min. After elimination of the fat layer, 50 ml of milk was filtered through Whatman filter paper (Millipore Corporation, Maid Stone, UK) and then passed through IAC at flow-rate of 1–2 drops s⁻¹. The column was washed with 20 ml of PBS (1–2 drops s⁻¹). Elution was performed with 3 ml of methanol. The elute was evaporated to dryness under stream of nitrogen. The residue was re-dissolved in 250 µl of HPLC mobile phase and injected into the HPLC system. For samples of powdered milk 5 g were suspended in 30 ml of distilled water, heated to 50°C, homogenized and then processed as for liquid milk.

Spiked samples. Samples spiked before extraction were used to check the performance of the extraction and clean-up procedure and to obtain validation parameters. Spiking solutions of mycotoxins were prepared daily by dilution with HPLC mobile phases. For samples of liquid

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3 milk, after thoroughly mixing for 30 min, the mycotoxins fortified milks were left for at least
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5 2 hours at room temperature to enable equilibration and used to assay the cleaning procedures
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7 prior to HPLC analysis. For samples of powdered milk, spiked samples were prepared by
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9 adding appropriate volume of the spiking solution of mycotoxins to the milk suspension
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11 (described above) and then processed as for liquid milk.
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15 16 **High-performance liquid chromatography**

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18 The chromatographic system consisted of Jasco880 pump and Jasco821 fluorescence detector
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20 (Jasco, Tokyo, Japan). JascoBorwin software was used for data processing. The excitation
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22 wavelength (λ_{ex}) and emission wavelength (λ_{em}) were set at 380 and 420 nm for OTA and at
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24 365 and 435 nm for AFM1, respectively. The reversed-phase column was a Luna C18 ODS2,
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26 3 μ m, (4.6 x 150mm) (Phenomenex® Torrance, CA, USA). The column was kept at room
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28 temperature. The HPLC was operated with mobile phase system consisting of methanol-
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30 phosphate buffer solution pH 7.5 (0.03 M Na₂HPO₄, 0.007M NaH₂PO₄) 50/50 % v/v at flow
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32 rate of 1 ml/min and acetonitrile-water 23/77 % v/v at flow rate of 1 ml/min, for OTA and
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34 AFM1, respectively. Calibration curves were based on the analysis of triplicate standards
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36 solution at 5 concentration levels in matrix and resulted in good linear relationship between
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38 peak area and mycotoxins concentration ($R^2 > 0.999$). The limits of detection (LOD) and
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40 quantitation (LOQ), calculated as threefold and tenfold the standard deviation of the
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42 intercepts of calibration curves are reported in table 1. The recovery (percentage of standard
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44 added to sample that is recovered after extraction and clean-up) of the extraction method was
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46 determined by sample fortification (Table 2). Intra-day reproducibility was evaluated by
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48 analyzing three milk samples with mycotoxins to obtain three different concentrations (10, 50,
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50 and 100 ng/L). Day-to-day reproducibility was determined by analysis of three replicate
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52 samples of the three concentrations (10, 50, and 100 ng/L) on seven consecutive days. From
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54 the data obtained, intra- and interday coefficient of deviations (CV) were calculated.
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Selectivity studies have been expressed as the ability to assess unequivocally mycotoxins in the presence of components which may be expected to be present: it has been evaluated by the comparison of free-mycotoxin milk samples vs spiked milk for both mycotoxins. Typical chromatograms obtained from mycotoxins free milk samples are reported in figure 1 and 2 and compared with that obtained from spiked samples with mycotoxins. Absence of any interfering peak at the retention times of OTA and AFM1 are clearly evident.

Statistical analysis

Values are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using two-tailed Student's *t*-test, Mann-Whitney test, Tukey test and one-way analysis of variance (ANOVA). Correlations between two parameters were determined by Pearson's correlation (*r*) coefficient analysis. Findings of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

A total of 185 infant cow's milk-based formula samples were analyzed. AFM1 was detected only in 2 samples (range 11.8-15.3 ng/L) from the same brand (n. = 10). The AFM1 contamination levels and incidence in infant formula marketed in Italy were lower than those observed in previous studies regarding milk analysis (Galvano et al., 1998; 2001). These results are probably due to the increased attention paid in European countries to the presence of aflatoxins in feeds and milk products and suggest that regulatory limits appear to be helpful.

OTA was detected in 133 (72%) samples (range 35.1-689.5 ng/L). The most contaminated one was a pre-term ready-to-use preparation containing 689.5 ng/L of OTA. In contrast to AFM1, no regulation for OTA in milk exists, even though it has been suggested that the level of this toxin in cow's milk may exceed the TDI of 5 ng/kg b.w. per day for small children in some areas in Norway. The European legislation limit of 0.50 ppb ($\mu\text{g/L}$) for

cereal-based infant foods was established (EC, 2006). Regarding OTA, Valenta and Goll (1996) did not find OTA in any of the 121 cow's milk samples analyzed from a northern region of Germany, while OTA was present in a small percentage (10-15 %) of milk samples in Sweden and Norway in the range of 10–50 ng/L (Skaug, 1999; Breitholtz-Emanuelsson et al., 1993). The reason of our data could be a higher contamination of feedstuffs in Italy or the existence of other possible routes of OTA exposure such as inhalation of airborne particles containing mycotoxins, as suggested by Skaug (1999). Furthermore, there can be other possible sources of OTA in samples other than the original milk used for manufacturing infant formulas. Different ingredients have been added to infant formulae not only to simulate better the composition of breast milk, but also to impart health benefits. Raw material (cow's milk or isolated protein), processing methods, additives or mineral supplements, and storage will in part determine the levels of minor and trace constituents present in the final product. In the present study, correlations analysis between the OTA contamination levels of different types of infant formula and their main characteristics (lipid, protein and carbohydrates contents) showed no significant ($p>0.05$) correlation between parameters.

It has been observed that OTA contamination was 80% in the ready-to-use preparations and 63% in the powdered samples. Pre-term and starter ready-to-use formula preparations showed the same incidence of positive samples (80%), while pre-term powdered formula showed 100% incidence and starter formula the 60%, although the numbers of pre-term powdered formula tested were insufficient to obtain an accurate picture of the incidence contamination (Table 4).

Non-parametric statistical analyses (Mann Whitney test) were performed with OTA content for comparison of the different types of formulas, not establishing significant differences ($p>0.05$) between different groups. The OTA content in both powder and ready-to-use formulas has been statistically evaluated to understand the influence of the aggregation

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state on final OTA level in formulas studied. OTA concentration found in 9 different formulas marked in both forms did not differ significantly, indicating that the most important factor of OTA levels was the formula ingredients. Furthermore, the present study shows a tendency for pre-term both powder and ready-to-use formula to have only slightly higher OTA levels than starter formulas.

Bar diagrams of OTA concentration distribution for all formulas commercialized by each brand analyzed (n. = 14) are shown in figures 3-4. A comparison of significant differences (ANOVA, $p=0.0182$) among ready-to-use preparations provided from the commercial brands was performed (Figure 3). Among the ready-to-use preparations, OTA level provided by brand 1 (n. = 15, 216.8 ± 217.4 ng/L, range 54.9-689.5 ng/L) was significantly higher (Tukey test, $p<0.05$) than those from most of the other brands. Regarding the powdered formulas, a comparison of significant differences (ANOVA, $p=0.0438$) among commercial brands was performed (Figure 4). OTA level provided by brand 1 (n. = 5, 195.2 ± 138.2 ng/L, range 65.4-340.5 ng/L) was significantly higher (Tukey test, $p<0.05$) than those from most of the other brands.

Non-parametric statistical analyses (Mann Whitney test) were performed with OTA content for comparison of the different types of formulas provided from the same commercial brand (pre-term vs. starter formula), not establishing significant differences ($p>0.05$) between different groups. The same statistical analyses were performed with OTA content for comparison of the different types of formulas provided from the same commercial brand (powdered vs. ready-to-use formula), not establishing significant differences ($p>0.05$) between different groups.

The OTA concentrations determined in the different types of formulas were used to calculate mean daily and weekly dietary OTA intakes by infants on the basis of feeding tables provided by brands, under the assumption that they receive only formulas. The mean daily

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3 intakes for ready-to-use pre-term and starter formula were 16.08 ± 3.93 and 12.29 ± 2.12 ng/kg
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5 b.w., respectively; while corresponding weekly intakes were 112.56 ± 27.51 and 86.03 ± 14.84
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7 ng/kg b.w., respectively. The mean daily and weekly intakes for powdered starter formula
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9 were 10.67 ± 1.95 and 74.69 ± 13.65 ng/kg b.w., respectively; while for powdered pre-term
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11 formula it has been impossible to calculate these values because of the few samples.
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15 The Scientific Committee for Food (SCF) reviewed the toxicology on OTA and
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17 concluded that it would be prudent to reduce exposure to OTA ensuring that exposures are
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19 towards the lower end of the range of tolerable daily intakes of 1.2-14 ng/kg b.w. per day.
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21 OTA was also evaluated by the Joint FAO/WHO Expert Committee on Food Additives
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23 (JECFA) and a provisional tolerable weekly intake (PTWI) of 100 ng/kg b.w. was established.
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25 In the present investigation, the average OTA levels in pre-term ready-to-use infant formula
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27 were sufficient to cause a higher intake of OTA than the suggested TDI and PTWI, while
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29 other formulations were below these limits.
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34 Interestingly, OTA was also detected in milk from healthy mothers in Germany,
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36 Norway, Sweden, Hungary, Italy, Australia and Sierra Leone (Scott, 2005). The present
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38 results are comparable with those obtained by Turconi et al. (2004) and Galvano et al. (2008)
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40 in two studies regarding human milk performed in Italy (198 of 231 samples contaminated
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42 with 1–57 ng/L and 74 of 82 samples contaminated with < 5 ng/L–405 ng/L, respectively),
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44 while are lower than those reported by Micco et al. (1991; 1995) (ranges 1700–6600 ng/L and
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46 100–12000 ng/L, respectively).
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50 Due to high toxicity and carcinogenic properties of OTA, its presence in milk is a
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52 concern. OTA is resistant to thermal inactivation and other varieties of food processing
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54 procedures, so to produce high quality milk, it is essential to keep feeds free from
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56 contamination by OTA. Mycotoxin concentrations in animal feed can be reduced by good
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58 manufacturing practice and good storage practices. On the other hand, infant formula
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manufacturers could try to obtain substantial reductions in levels of contaminants, presumably through more careful selection of raw materials and/or avoidance of contamination during industrial processing.

In conclusion, the results of this study firstly indicate the OTA presence in infant cow's milk-based formulas and therefore, they point out the need to perform occurrence surveys in this area. The present study also suggests the usefulness of prevention programs especially when attempting to identify risk markers of the infant feed quality. Analysis of infant cow's milk-based formulas samples hence may serve as a marker of exposure to OTA. At the same time, OTA levels in milk indicate the exposure of new-born infants, which needs to be considered in the overall risk characterization.

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FIGURE LEGENDS

Figure 1. Chromatograms obtained from extracts of (a) a blank milk sample and (b) a milk sample spiked with AFM1.

Figure 2. Chromatograms obtained from extracts of (a) a blank milk sample and (b) a milk sample spiked with OTA.

Figure 3. OTA levels (ng/L) in the ready-to-use milk-based infant formulas provided by different manufacturers.

Figure 4. OTA levels (ng/L) in the powdered milk-based infant formulas provided by different manufacturers.

Brand	Type	Lipids	Proteins	Carbohydrates	Minerals
5	Pre-term	3.75	2.03	8.32	0.37
1	Pre-term	4.80	3.20	10.90	0.50
1	Pre-term	3.80	2.20	10.10	0.40
2	Pre-term	4.40	2.40	7.70	0.34
3	Pre-term	4.20	2.30	8.60	0.47
4	Pre-term	4.20	2.20	8.30	0.36
4	Pre-term	4.00	2.00	7.80	0.35
6	Pre-term	4.00	2.00	7.80	0.35
6	Pre-term	4.00	2.30	7.50	0.32
7	Pre-term	4.40	2.30	8.30	0.400
2	Starter	3.60	1.40	7.40	0.22
4	Starter	4.10	1.40	7.30	0.30
6	Starter	3.70	1.40	7.50	0.25
3	Starter	3.60	1.20	7.50	0.25
8	Starter	3.60	1.40	7.70	0.30
9	Starter	3.60	1.30	7.70	0.30
4	Starter	3.50	1.70	7.20	0.22
5	Starter	3.50	1.50	8.30	0.32
1	Starter	3.10	1.80	8.30	0.30
7	Starter	3.70	1.30	7.70	0.30

Table 1. Composition of liquid (ready-to-use) infant formula analyzed, (values are expressed as g/100 ml of product).

Brand	Type		Lipids	Proteins	Carbohydrates	Minerals
2	Pre-term	100 g of powd.	0	7.60	71.5	3.30
		4.2 %/100 ml	0	0.30	3.00	0.14
1	Starter	100 g of powd.	22.3	12.9	59.6	2.20
		13.9%/100 ml	3.10	1.80	8.30	0.30
10	Starter	100 g of powd.	26.5	11.2	57.3	1.90
		13.5%/100 ml	3.60	1.50	7.70	0.27
11	Starter	100 g of powd.	25.9	10.4	55.5	1.50
		13.5%/100 ml	3.50	1.40	7.50	0.20
12	Starter	100 g of powd.	28.1	13.6	53.3	2.00
		12.9%/100 ml	3.60	1.70	6.90	0.25
13	Starter	100 g of powd.	23.6	11.0	60.3	1.90
		13.5%/100 ml	3.30	1.50	8.40	0.27
14	Starter	100 g of powd.	25.9	12.5	57.1	1.80
		13.5%/100 ml	3.50	1.70	7.70	0.22
2	Starter	100 g of powd.	25.6	10.2	52.9	1.60
		13.9%/100 ml	3.60	1.40	7.30	0.22
9	Starter	100 g of powd.	26.7	9.60	57	2.20
		13.5%/100 ml	3.60	1.30	7.70	0.30
4	Starter	100 g of powd.	25.9	12.5	53.0	1.55
		13.5%/100 ml	3.50	1.70	7.20	0.21
8	Starter	100 g of powd.	26.7	10.4	57.0	2.20
		13.5%/100 ml	3.60	1.40	7.70	0.30
7	Starter	100 g of powd.	27.5	9.60	57.0	2.20
		13.5%/100 ml	3.70	1.30	7.70	0.30
5	Starter	100 g of powd.	24.8	10.8	59.60	2.30
		14%/100 ml	3.47	1.51	8.34	0.32
7	Starter	100 g of powd.	27.5	9.60	57.2	2.20
		13.5%/100 ml	3.70	1.30	7.70	0.30
2	Starter	100 g of powd.	25.6	12.8	56.5	2.20
		13.3%/100 ml	3.40	1.70	7.50	0.30
5	Starter	100 g of powd.	23.0	12.50	59.0	3.00
		14%/100 ml	3.22	1.75	8.26	0.42
3	Starter	100 g of powd.	27.7	9.50	57.9	1.90
		12.9%/100 ml	3.60	1.20	7.50	0.24

Table 2. Composition of powder infant formula analyzed.

AFM1					
Matrix	LOD	LOQ	Recovery % $\pm$ SD	CV% intra-day	CV% inter-day
Liquid	2.5 ng/L	5 ng/L	75.0 $\pm$ 0.3%	3	8
Powders			69.5 $\pm$ 0.2%	7	9
OA					
Matrix	LOD	LOQ	Recovery % $\pm$ SD	CV% intra-day	CV% inter-day
Liquid	1.25 ng/L	6.25 ng/L	87.1 $\pm$ 0.3%	2	7
Powders			79.5 $\pm$ 0.5%	3	8

Table 3. Validation parameters for HPLC methods of AFM1 and OA, (LOD, limit of detection; LOQ, limit of quantitation; SD, standard deviation; CV, coefficient of variation).

Infant formula	Number of samples	Numbers of positives (%)	Mean±SE	Range
Pre-term	55	45 (80%)	106.2±23.88 ng/L	<LOD-689.5 ng/L
<u>Liquid</u> <u>Powder</u>	50	40 (80%)	107.2±26.26 ng/L	<LOD-689.5 ng/L
	5	5 (100%)	96.10±15.59 ng/L	80.49-127.3 ng/L
Starter	130	88 (68%)	69.77±8.81 ng/L	<LOD-355.8 ng/L
<u>Liquid</u> <u>Powder</u>	50	40 (80%)	65.57±11.33 ng/L	<LOD-299.1 ng/L
	80	48 (60%)	72.40±12.52 ng/L	<LOD-355.8 ng/L

Table 4. OA concentrations in different types of infant formulas investigated, (SE, standard error of the mean; LOD, limit of detection).

Figure Legends

Figure 1. Chromatograms obtained from extracts of (a) a blank milk sample and (b) a milk sample spiked with AFM1.

Figure 2. Chromatograms obtained from extracts of (a) a blank milk sample and (b) a milk sample spiked with OTA.

Figure 3. OTA levels (ng/L) in the ready-to-use milk-based infant formulas provided by different manufacturers.

Figure 4. OTA levels (ng/L) in the powdered milk-based infant formulas provided by different manufacturers.

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Figure 1

Figure 2

Figure 3

Figure 4