



HAL
open science

MYCOTOXIN DETECTION IN INFANT FORMULA MILKS IN ITALY

Valentina Meucci, Elisabetta Razzuoli, Giulio Soldani, Francesco Massart

► **To cite this version:**

Valentina Meucci, Elisabetta Razzuoli, Giulio Soldani, Francesco Massart. MYCOTOXIN DETECTION IN INFANT FORMULA MILKS IN ITALY. Food Additives and Contaminants, 2009, 27 (01), pp.64-71. 10.1080/02652030903207201 . hal-00573904

HAL Id: hal-00573904

<https://hal.science/hal-00573904v1>

Submitted on 5 Mar 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



MYCOTOXIN DETECTION IN INFANT FORMULA MILKS IN ITALY

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2009-054.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	17-Jul-2009
Complete List of Authors:	meucci, valentina; University of Pisa, Veterinary Clinics razzuoli, elisabetta; University of Pisa, Veterinary Clinics soldani, giulio; University of Pisa, Veterinary Clinics massart, francesco; University of Pisa, Pediatrics
Methods/Techniques:	Chromatography - HPLC, Clean-up - affinity columns
Additives/Contaminants:	Mycotoxins, Mycotoxins – aflatoxins, Mycotoxins – ochratoxin A
Food Types:	Baby food, Infant formulae, Milk

SCHOLARONE™
Manuscripts

MYCOTOXIN DETECTION IN INFANT FORMULA MILKS IN ITALY

Valentina Meucci^{a*}, Elisabetta Razzuoli^a, Giulio Soldani^a, Francesco Massart^b.

Departments of ^aVeterinary Clinics, and ^bPediatrics, University of Pisa, 56125 Pisa, Italy

**To whom correspondence should be addressed:*

Valentina Meucci,

Department of Veterinary Clinics

Viale delle Piagge 2

56125 PISA

Italy

Tel.: (+39) 050-2216-803

Fax: (+39) 050-2216-813

E-mail: vmeucci@vet.unipi.it

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

1
2
3 grown in Europe (EC, 2003b; EFSA, 2004). The emergence of aflatoxins in the European
4
5 continent is concurrent with the increase in the annual average temperature registered in the
6
7 past decade (Anderson et al., 2004).
8
9

10
11 Investigations on milk AFM1 contamination are regularly conducted in European
12
13 countries (EFSA, 2004), but there is little information on the milk contamination by other
14
15 mycotoxins. In particular, there is scarce information on the presence of OTA in cow's milk
16
17 (Skaug, 1999; Breitholtz-Emanuelsson et al., 1993; Valenta and Goll, 1996), and no data are
18
19 available in Italy concerning infant milk-based formula contamination by this mycotoxin.
20
21 Consumption of cow's milk has not previously been considered as possible source of OTA
22
23 intake, because of the microflora in the rumen hydrolyses OTA to the less toxic metabolite
24
25 ochratoxin α (Hult et al., 1976; Sreemannarayana et al., 1988). However, OTA has been
26
27 found in bovine milk, in low concentrations, indicating that small amounts may escape rumen
28
29 metabolism and are absorbed (Skaug, 1999; Breitholtz-Emanuelsson et al., 1993). The OTA
30
31 concentration in cow's milk is expected to be very low, but even low concentrations might
32
33 markedly increase OTA daily intake by consumers of large quantities of milk such as
34
35 children, and contribute to significant portion of the total dietary intake (Skaug, 1999).
36
37
38
39
40

41
42 After birth, infant formulas constitute an important or often sole source of food for
43
44 newborns and infants during the first months of life. Thus, these formulas have a special role
45
46 in the infant diet because they should serve as substitutes for human milk. It is desirable that
47
48 infant formulas should be proportionally similar or inferior in AFM1 and OTA levels to those
49
50 found by human milk. In general, AFM1 and OTA concentrations in breast milk show marked
51
52 variability depending on mother's life style and dietary habits. OTA was detected in milk
53
54 from healthy mothers in Germany, Norway, Sweden, Hungary, Italy, Australia and Sierra
55
56 Leone (Scott, 2005). Previous data from Italy had indicated a high prevalence of OTA in
57
58 human milk samples with concentrations ranging between 1700-6600 ng/L and 100-12000
59
60

1
2
3 ng/L, respectively (Micco et al., 1991; 1995). A second study has been carried out in Italy
4
5 (Turconi et al., 2004). OTA was detected in 198 samples (85.7%) at an average value of 6.01
6
7 ng/L. More recently, data from milk samples collected from 82 Italian women indicated that
8
9 74% of human milk samples contained OTA in the range < 5 ng/L-405 ng/L (Galvano et al.,
10
11 2008). In the same study AFM1 was detected in four (5%) of milk samples (ranging from < 7
12
13 ng/L to 140 ng/L) (Galvano et al., 2008). Relatively little work on the topic of mycotoxins in
14
15 infant food has been reported in the literature (Galvano et al., 1998; 2001; Lombaert et al.,
16
17 2003; Tam et al., 2006; Beretta et al., 2002). Most data have only been part of larger surveys
18
19 of cereal foods (Lombaert et al., 2003; Tam et al., 2006; Beretta et al., 2002).
20
21
22
23
24

25 For these reasons, the aim of the following work was to determine AFM1 and OTA
26
27 levels in the leading brands of infant formulas marketed in Italy, and then, to assess their
28
29 repercussion in the provisional tolerable weekly intake of OTA and of AFM1.
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 µg/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 µg/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

1
2
3 milk, after thoroughly mixing for 30 min, the mycotoxins fortified milks were left for at least
4
5
6 2 hours at room temperature to enable equilibration and used to assay the cleaning procedures
7
8 prior to HPLC analysis. For samples of powdered milk, spiked samples were prepared by
9
10 adding appropriate volume of the spiking solution of mycotoxins to the milk suspension
11
12 (described above) and then processed as for liquid milk.
13
14

15 16 **High-performance liquid chromatography**

17
18 The chromatographic system consisted of Jasco880 pump and Jasco821 fluorescence detector
19
20 (Jasco, Tokyo, Japan). JascoBorwin software was used for data processing. The excitation
21
22 wavelength (λ_{ex}) and emission wavelength (λ_{em}) were set at 380 and 420 nm for OTA and at
23
24 365 and 435 nm for AFM1, respectively. The reversed-phase column was a Luna C18 ODS2,
25
26 3 μm , (4.6 x 150mm) (Phenomenex® Torrance, CA, USA). The column was kept at room
27
28 temperature. The HPLC was operated with mobile phase system consisting of methanol-
29
30 phosphate buffer solution pH 7.5 (0.03 M Na_2HPO_4 , 0.007M NaH_2PO_4) 50/50 % v/v at flow
31
32 rate of 1 ml/min and acetonitrile-water 23/77 % v/v at flow rate of 1 ml/min, for OTA and
33
34 AFM1, respectively. Calibration curves were based on the analysis of triplicate standards
35
36 solution at 5 concentration levels in matrix and resulted in good linear relationship between
37
38 peak area and mycotoxins concentration ($R^2 > 0.999$). The limits of detection (LOD) and
39
40 quantitation (LOQ), calculated as threefold and tenfold the standard deviation of the
41
42 intercepts of calibration curves are reported in table 1. The recovery (percentage of standard
43
44 added to sample that is recovered after extraction and clean-up) of the extraction method was
45
46 determined by sample fortification (Table 2). Intra-day reproducibility was evaluated by
47
48 analyzing three milk samples with mycotoxins to obtain three different concentrations (10, 50,
49
50 and 100 ng/L). Day-to-day reproducibility was determined by analysis of three replicate
51
52 samples of the three concentrations (10, 50, and 100 ng/L) on seven consecutive days. From
53
54 the data obtained, intra- and interday coefficient of deviations (CV) were calculated.
55
56
57
58
59
60

1
2
3 Selectivity studies have been expressed as the ability to assess unequivocally mycotoxins in
4 the presence of components which may be expected to be present: it has been evaluated by the
5 comparison of free-mycotoxin milk samples vs spiked milk for both mycotoxins. Typical
6 chromatograms obtained from mycotoxins free milk samples are reported in figure 1 and 2
7 and compared with that obtained from spiked samples with mycotoxins. Absence of any
8 interfering peak at the retention times of OTA and AFM1 are clearly evident.
9
10
11
12
13
14
15
16

17 **Statistical analysis**

18
19 Values are expressed as mean \pm standard deviation (SD). Statistical analysis was
20 performed using two-tailed Student's *t*-test, Mann-Whitney test, Tukey test and one-way
21 analysis of variance (ANOVA). Correlations between two parameters were determined by
22 Pearson's correlation (*r*) coefficient analysis. Findings of $p < 0.05$ were considered significant.
23
24
25
26
27
28
29
30
31

32 **RESULTS AND DISCUSSION**

33
34 A total of 185 infant cow's milk-based formula samples were analyzed. AFM1 was
35 detected only in 2 samples (range 11.8-15.3 ng/L) from the same brand (n. = 10). The AFM1
36 contamination levels and incidence in infant formula marketed in Italy were lower than those
37 observed in previous studies regarding milk analysis (Galvano et al., 1998; 2001). These
38 results are probably due to the increased attention paid in European countries to the presence
39 of aflatoxins in feeds and milk products and suggest that regulatory limits appear to be
40 helpful.
41
42
43
44
45
46
47
48
49
50

51 OTA was detected in 133 (72%) samples (range 35.1-689.5 ng/L). The most
52 contaminated one was a pre-term ready-to-use preparation containing 689.5 ng/L of OTA. In
53 contrast to AFM1, no regulation for OTA in milk exists, even though it has been suggested
54 that the level of this toxin in cow's milk may exceed the TDI of 5 ng/kg b.w. per day for small
55 children in some areas in Norway. The European legislation limit of 0.50 ppb ($\mu\text{g/L}$) for
56
57
58
59
60

1
2
3 cereal-based infant foods was established (EC, 2006). Regarding OTA, Valenta and Goll
4 (1996) did not find OTA in any of the 121 cow's milk samples analyzed from a northern
5 region of Germany, while OTA was present in a small percentage (10-15 %) of milk samples
6 in Sweden and Norway in the range of 10–50 ng/L (Skaug, 1999; Breitholtz-Emanuelsson et
7 al., 1993). The reason of our data could be a higher contamination of feedstuffs in Italy or the
8 existence of other possible routes of OTA exposure such as inhalation of airborne particles
9 containing mycotoxins, as suggested by Skaug (1999). Furthermore, there can be other
10 possible sources of OTA in samples other than the original milk used for manufacturing infant
11 formulas. Different ingredients have been added to infant formulae not only to simulate better
12 the composition of breast milk, but also to impart health benefits. Raw material (cow's milk
13 or isolated protein), processing methods, additives or mineral supplements, and storage will in
14 part determine the levels of minor and trace constituents present in the final product. In the
15 present study, correlations analysis between the OTA contamination levels of different types
16 of infant formula and their main characteristics (lipid, protein and carbohydrates contents)
17 showed no significant ($p>0.05$) correlation between parameters.
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37

38 It has been observed that OTA contamination was 80% in the ready-to-use preparations
39 and 63% in the powdered samples. Pre-term and starter ready-to-use formula preparations
40 showed the same incidence of positive samples (80%), while pre-term powdered formula
41 showed 100% incidence and starter formula the 60%, although the numbers of pre-term
42 powdered formula tested were insufficient to obtain an accurate picture of the incidence
43 contamination (Table 4).
44
45
46
47
48
49
50
51

52 Non-parametric statistical analyses (Mann Whitney test) were performed with OTA
53 content for comparison of the different types of formulas, not establishing significant
54 differences ($p>0.05$) between different groups. The OTA content in both powder and ready-
55 to-use formulas has been statistically evaluated to understand the influence of the aggregation
56
57
58
59
60

1
2
3 state on final OTA level in formulas studied. OTA concentration found in 9 different formulas
4
5 marked in both forms did not differ significantly, indicating that the most important factor of
6
7 OTA levels was the formula ingredients. Furthermore, the present study shows a tendency for
8
9 pre-term both powder and ready-to-use formula to have only slightly higher OTA levels than
10
11 starter formulas.
12
13

14
15 Bar diagrams of OTA concentration distribution for all formulas commercialized by
16
17 each brand analyzed (n. = 14) are shown in figures 3-4. A comparison of significant
18
19 differences (ANOVA, $p=0.0182$) among ready-to-use preparations provided from the
20
21 commercial brands was performed (Figure 3). Among the ready-to-use preparations, OTA
22
23 level provided by brand 1 (n. = 15, 216.8 ± 217.4 ng/L, range 54.9-689.5 ng/L) was
24
25 significantly higher (Tukey test, $p<0.05$) than those from most of the other brands. Regarding
26
27 the powdered formulas, a comparison of significant differences (ANOVA, $p=0.0438$) among
28
29 commercial brands was performed (Figure 4). OTA level provided by brand 1 (n. = 5,
30
31 195.2 ± 138.2 ng/L, range 65.4-340.5 ng/L) was significantly higher (Tukey test, $p<0.05$) than
32
33 those from most of the other brands.
34
35
36
37

38
39 Non-parametric statistical analyses (Mann Whitney test) were performed with OTA
40
41 content for comparison of the different types of formulas provided from the same commercial
42
43 brand (pre-term vs. starter formula), not establishing significant differences ($p>0.05$) between
44
45 different groups. The same statistical analyses were performed with OTA content for
46
47 comparison of the different types of formulas provided from the same commercial brand
48
49 (powdered vs. ready-to-use formula), not establishing significant differences ($p>0.05$)
50
51 between different groups.
52
53
54

55
56 The OTA concentrations determined in the different types of formulas were used to
57
58 calculate mean daily and weekly dietary OTA intakes by infants on the basis of feeding tables
59
60 provided by brands, under the assumption that they receive only formulas. The mean daily

1
2
3 intakes for ready-to-use pre-term and starter formula were 16.08 ± 3.93 and 12.29 ± 2.12 ng/kg
4
5 b.w., respectively; while corresponding weekly intakes were 112.56 ± 27.51 and 86.03 ± 14.84
6
7 ng/kg b.w., respectively. The mean daily and weekly intakes for powdered starter formula
8
9 were 10.67 ± 1.95 and 74.69 ± 13.65 ng/kg b.w., respectively; while for powdered pre-term
10
11 formula it has been impossible to calculate these values because of the few samples.
12
13

14
15 The Scientific Committee for Food (SCF) reviewed the toxicology on OTA and
16
17 concluded that it would be prudent to reduce exposure to OTA ensuring that exposures are
18
19 towards the lower end of the range of tolerable daily intakes of 1.2-14 ng/kg b.w. per day.
20
21 OTA was also evaluated by the Joint FAO/WHO Expert Committee on Food Additives
22
23 (JECFA) and a provisional tolerable weekly intake (PTWI) of 100 ng/kg b.w. was established.
24
25 In the present investigation, the average OTA levels in pre-term ready-to-use infant formula
26
27 were sufficient to cause a higher intake of OTA than the suggested TDI and PTWI, while
28
29 other formulations were below these limits.
30
31
32

33
34 Interestingly, OTA was also detected in milk from healthy mothers in Germany,
35
36 Norway, Sweden, Hungary, Italy, Australia and Sierra Leone (Scott, 2005). The present
37
38 results are comparable with those obtained by Turconi et al. (2004) and Galvano et al. (2008)
39
40 in two studies regarding human milk performed in Italy (198 of 231 samples contaminated
41
42 with 1–57 ng/L and 74 of 82 samples contaminated with < 5 ng/L–405 ng/L, respectively),
43
44 while are lower than those reported by Micco et al. (1991; 1995) (ranges 1700–6600 ng/L and
45
46 100–12000 ng/L, respectively).
47
48
49

50
51 Due to high toxicity and carcinogenic properties of OTA, its presence in milk is a
52
53 concern. OTA is resistant to thermal inactivation and other varieties of food processing
54
55 procedures, so to produce high quality milk, it is essential to keep feeds free from
56
57 contamination by OTA. Mycotoxin concentrations in animal feed can be reduced by good
58
59 manufacturing practice and good storage practices. On the other hand, infant formula
60

1
2
3 manufacturers could try to obtain substantial reductions in levels of contaminants, presumably
4
5 through more careful selection of raw materials and/or avoidance of contamination during
6
7 industrial processing.
8
9

10 In conclusion, the results of this study firstly indicate the OTA presence in infant cow's
11
12 milk-based formulas and therefore, they point out the need to perform occurrence surveys in
13
14 this area. The present study also suggests the usefulness of prevention programs especially
15
16 when attempting to identify risk markers of the infant feed quality. Analysis of infant cow's
17
18 milk-based formulas samples hence may serve as a marker of exposure to OTA. At the same
19
20 time, OTA levels in milk indicate the exposure of new-born infants, which needs to be
21
22 considered in the overall risk characterization.
23
24
25
26
27
28

29 REFERENCES

- 30
31
32 Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P. 2004.
33
34 Emerging infectious diseases of plants: Pathogen pollution, climate change and agriculture
35
36 drivers. *Trends in Ecology & Evolution* 19: 536-544.
37
38
39 Beretta B, De Domenico R, Gaiaschi A, Ballabio C, Galli CL, Gigliotti C, Restani P. 2002.
40
41 Ochratoxin A in cereal-based baby foods: occurrence and safety evaluation. *Food Additives*
42
43 *and Contaminants* 19: 70-75.
44
45
46 Breitholtz-Emanuelsson A, Olsen M, Oskarsson A, Palminger I, Hult K. 1993. Ochratoxin A
47
48 in cow's milk and in human milk with corresponding human blood samples. *The Journal of*
49
50 *AOAC International* 76: 842-846.
51
52
53 Ceovic S, Hrabar A, Saric M. 1992. Epidemiology of Balkan endemic nephropathy. *Food and*
54
55 *Chemical Toxicology* 30: 183-188.
56
57
58
59
60

1
2
3 EC. 2003a. Commission Regulation (EC) No 2174/2003 of 12 December 2003 amending
4
5 Regulation (EC) No 466/2001 as regards aflatoxins. Official Journal of the European Union
6
7 L326: 12.
8

9
10 EC. 2003b. Commission Directive 2003/100/EC of 31 October 2003 amending Annex I to
11
12 Directive 2002/32/EC of the European Parliament and of the Council on undesirable
13
14 substances in animal feed. Official Journal of the European Union L285: 33.
15

16
17 EC. 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting
18
19 maximum levels for certain contaminants in foodstuffs. Official Journal of the European
20
21 Union L364: 5.
22

23
24 EFSA, 2006. Opinion of the scientific panel on contaminants in the food chain on a request
25
26 from the commission related to ochratoxin a in food. The EFSA Journal 365: 1-56.
27

28
29 EFSA. 2004. Opinion of the scientific panel on contaminants in the food chain on a request
30
31 from the commission related to aflatoxin B1 as undesirable substance in animal feed. The
32
33 EFSA Journal 39: 1-27.
34

35
36 FAO/WHO, 2001. Ochratoxin A. In: Safety evaluation of certain mycotoxins in food,
37
38 Prepared by the 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives
39
40 (JECFA). WHO Food Additives Series 47, pp 281–387. World Health Organisation, Geneva,
41
42 Switzerland.
43

44
45 Galvano F, Galofaro V, de Angelis A, Galvano M, Bognanno M, Galvano G. 1998. Survey of
46
47 the occurrence of aflatoxin M1 in dairy products marketed in Italy. Journal of Food Protection
48
49 61: 738-741.
50

51
52 Galvano F, Galofaro V, Ritieni A, Bognanno M, De Angelis A, Galvano G. 2001. Survey of
53
54 the occurrence of aflatoxin M1 in dairy products marketed in Italy: second year of
55
56 observation. Food Additives and Contaminants 18: 644-646.
57
58
59
60

- 1
2
3 Galvano F, Pietri A, Bertuzzi T, Gagliardi L, Ciotti S, Luisi S, Bognanno M, La Fauci L,
4
5 Iacopino AM, Nigro F, Li Volti G, Vanella L, Giammanco G, Tina GL, Gazzolo D. 2008.
6
7 Maternal dietary habits and mycotoxin occurrence in human mature milk. *Molecular and*
8
9 *Nutritional Food Research* 52: 496-501.
- 10
11
12 Hult K, Teiling A, Gatenbeck S. 1976. Degradation of ochratoxin A by a ruminant. *Applied*
13
14 *and Environmental Microbiology* 32: 443-444.
- 15
16
17 IARC. 1993a. Aflatoxins. Some naturally occurring substances: food items and constituents,
18
19 heterocyclic aromatic amines and mycotoxins. *IARC Monographs on the Evaluation of*
20
21 *Carcinogenic Risks to Humans*, vol. 56, Lyon, France, pp. 245-395.
- 22
23
24 IARC. 1993b. Ochratoxin A. Some naturally occurring substances; food items and
25
26 constituents, heterocyclic aromatic amines and mycotoxins, *IARC Monographs on the*
27
28 *Evaluation of Carcinogenic Risks to Humans*, vol. 56, Lyon, France, pp. 489-521.
- 29
30
31 Krogh P. 1987. *Mycotoxins in Food*. Food Science and Technology. New-York, NY, USA:
32
33 Academic Press. Ochratoxins in food; p. 97-121.
- 34
35
36 Krogh P. 1992. Role of ochratoxin in disease causation. *Food and Chemical Toxicology* 30:
37
38 213-224.
- 39
40
41 Lombaert GA, Pellaers P, Roscoe V, Mankotia M, Neil R, Scott PM. 2003. Mycotoxins in
42
43 infant cereal foods from the Canadian retail market. *Food Additives and Contaminants* 20:
44
45 494-504.
- 46
47
48 Micco C, Ambruzzi MA, Miraglia M, Brera C, Onori R, Benelli L. 1991. Contamination of
49
50 human milk with ochratoxin A. *IARC scientific publications* 115: 105-108.
- 51
52
53 Micco C, Miraglia M, Brera C, Corneli S, Ambruzzi A. 1995. Evaluation of ochratoxin A in
54
55 human milk in Italy. *Food Additives and Contaminants* 12: 351-354.
- 56
57
58
59
60

- 1
2
3 SCF. 1998. Opinion on of the Scientific Committee on Food (SCF) on ochratoxin A.
4
5 Expressed on 17 September 1998. Available at:
6
7 http://europa.eu.int/comm/food/fs/sc/scf/out14_en.html Accessed November 15, 2008.
8
9
10 Scott PM. 2005. Biomarkers of human exposure to ochratoxin A. Food Additives and
11
12 Contaminants 22: 99-107.
13
14 Skaug MA. 1999. Analysis of Norwegian milk and infant formulas for ochratoxin A. Food
15
16 Additives and Contaminants 16: 75-78.
17
18
19 Sreemannarayana O, Frohlich AA, Vitti TG, Marquardt RR, Abramson D. 1988. Studies of
20
21 the tolerance and disposition of ochratoxin A in young calves. Animal Science 66: 1703-
22
23 1711.
24
25
26 Tam J, Mankotia M, Mably M, Pantazopoulos P, Neil RJ, Calway P, Scott PM. 2006. Survey
27
28 of breakfast and infant cereals for aflatoxins B1, B2, G1 and G2. Food Additives and
29
30 Contaminants 23: 693-699.
31
32
33 Turconi G, Guarcello M, Livieri C, Comizzoli S, Maccarini L, Castellazzi AM, Pietri A, Piva
34
35 G, Roggi C. 2004. Evaluation of xenobiotics in human milk and ingestion by the newborn--an
36
37 epidemiological survey in Lombardy (Northern Italy). European Journal of Nutrition 43: 191-
38
39 197.
40
41
42 Valenta H, Goll M. 1996. Determination of ochratoxin A in regional samples of cow's milk
43
44 from Germany. Food Additives and Contaminants 13: 669-676.
45
46
47 Valenta H. 1998. Chromathographic methods for the determination of ochratoxin A in animal
48
49 and human tissues and fluids. Journal of Chromathography A 815: 75-92.
50
51
52
53
54
55
56
57
58
59
60

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>	50	40 (80%)	107.2±26.26 ng/L	<LOD-689.5 ng/L
<u>Powder</u>	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>	50	40 (80%)	65.57±11.33 ng/L	<LOD-299.1 ng/L
<u>Powder</u>	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.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

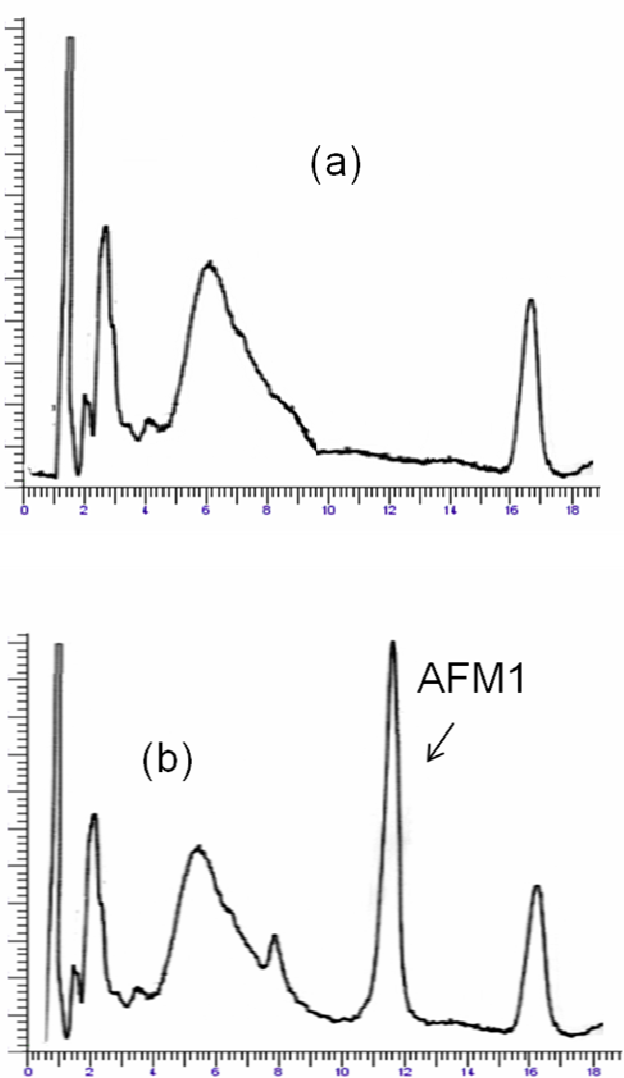
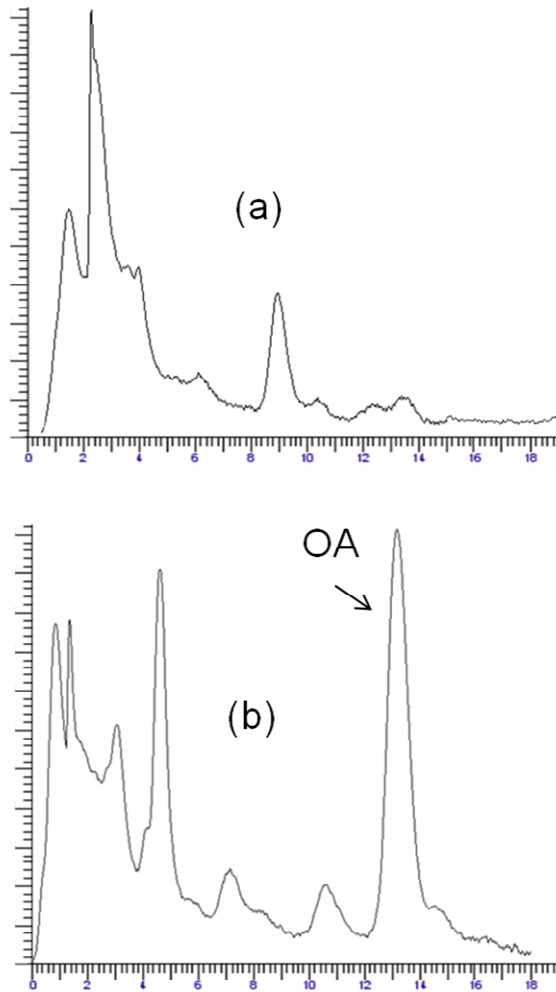


Figure 1

**Figure 2**

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

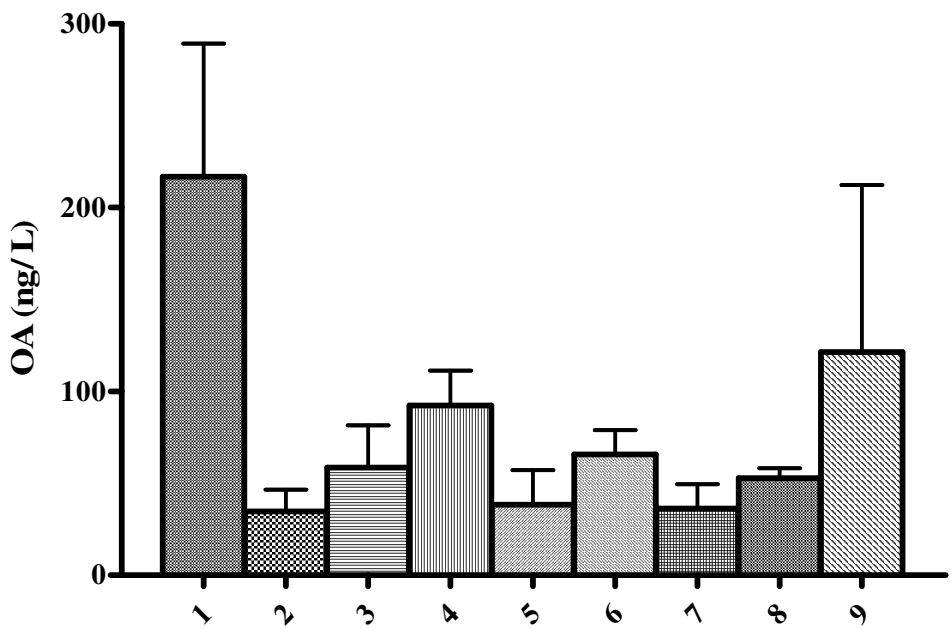


Figure 3

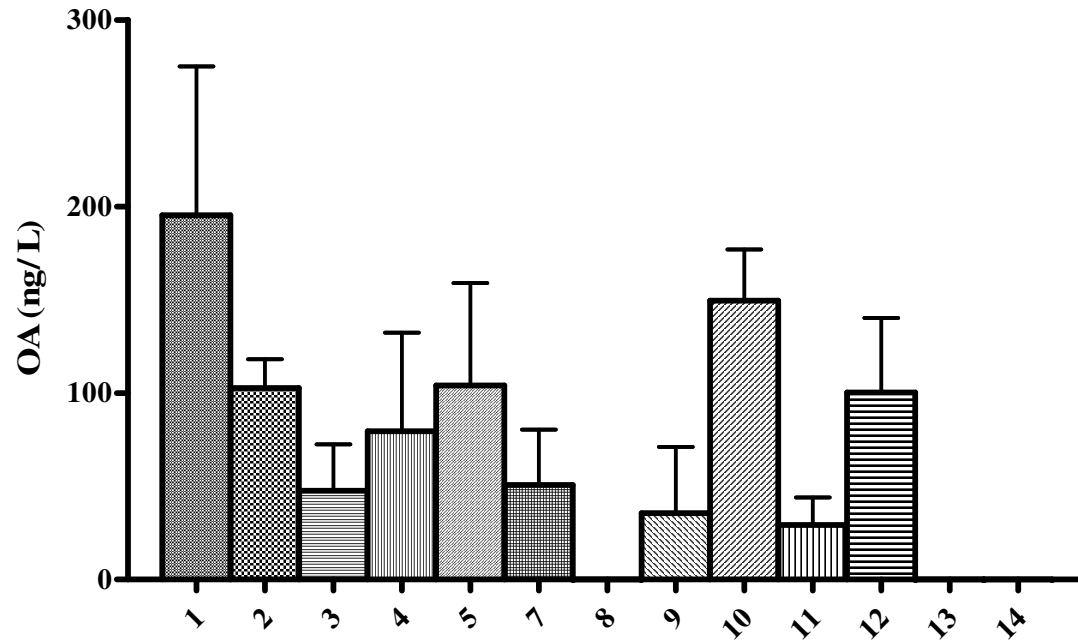


Figure 4