Aflatoxins content and risk assessment from Spanish infant cereals

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Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) are immunosuppressant, mutagenic, teratogenic and carcinogenic agents with a widespread presence in foodstuffs. Since human exposure to aflatoxins occurs primarily by contaminated food intake, and given the greater susceptibility of infants to their adverse effects, the quantification of these mycotoxins in infant food based on cereals is of relevance. Aflatoxins levels were determined in ninety-one Spanish infant cereals classified in terms of non- and organically produced and several types from ten different manufactures, using a extraction procedure followed by inmunoaffinity column clean-up step and High Pressure Liquid Chromatography (HPLC) with fluorescence detection (FLD) and post-column derivatization (Kobra Cell system). Daily aflatoxin intake was also assessed. The preliminary analysis revealed a noticeable detected infant cereal samples for total aflatoxin (81 %), corresponding to a 64 %, 39 %, 65 % and 43 % for AFB_1, AFB_2, AFG_1 and AFG_2. Lower aflatoxin values (median, Q1;Q3) in conventional infant cereal (n=74, AFB_1: <LOD (n.d.;0.019), AFB_2: n.d. (n.d;0.011), AFG_1: <LOD (n.d.;0.004), and AFG_2: n.d. (n.d.;<LOD) and AFtotal: 0.014 (<LOD;0.035 µg kg^{-1}) in comparison with infant cereal ecologically produced (n=17, AFB_1: 0.022 (0.016;0.212), AFB_2: n.d. (n.d;0.027), AFG_1: 0.020 (0.014;0.053), and AFG_2: 0.007 (n.d.;0.024) and total AF: 0.047 (0.030;0.311 µg kg^{-1}) were found. In addition, five organic formulations (3.112, 1.981, 0.943, 0.471 and 0.212 µg kg^{-1}) exceeded European AFB_1 legislation (0.10 µg kg^{-1}) versus two conventional cereals (0.346 and 0.117 µg kg^{-1}). According to the type of infant cereal, cereals with cocoa provided the highest aflatoxin levels, gluten-free and cereals with dehydrated fruits were in a intermediate level and milk- or honey- based cereals and multicereals contained the lowest levels. With the exception of non-compliant cocoa based organic formulation, none of the infant cereals analyzed provides a higher intake of 1 ng kg^{-1} body weight per day, suggesting that infants fed on infant cereals are exposed to low health hazard. Nevertheless, manufactures are called for continued efforts to routinely monitor and more careful selection of raw material to minimize aflatoxin levels in these infant foods.

Keywords: aflatoxins; infant cereals; daily intake; food analysis; HPLC

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Introduction

Aflatoxins are a type of mycotoxins produced by certain species of Aspergillus, particularly A. flavus, A. parasiticus and A. nomius. There are more than twenty distinct, but structurally related aflatoxin compounds, the four most commonly seen are known as aflatoxin B_1 (AFB$_1$), aflatoxin B_2 (AFB$_2$), aflatoxin G_1 (AFG$_1$) and aflatoxin G_2 (AFG$_2$) (Nachtmann et al. 2007; Cavaliere et al. 2007; Tam et al. 2006).

Tropical and subtropical regions (warm and humid weather), provide optimal conditions for fungal growth (Cavaliere et al. 2007, Zöllner and Mayer-Helm 2006, Zinedine et al. 2006, Aycicek et al. 2005, Ramos-Catharino et al. 2005, Shenasi et al. 2002, Hussein and Brasel 2001). The incidence of aflatoxins is enhanced by factors such as stress or damage to the plant due to drought before harvest, insect activity, soil type and inadequate storage conditions (Cavaliere et al. 2007). Therefore, species contamination with aflatoxins may occur during the crop, processing (especially drying), transformation or storage (Sforza et al. 2006, Bircan et al. 2005, Hussein and Brasel 2001).

Aflatoxins are frequently found in many foodstuffs for humans or animals (Ali et al. 2005, Nasir and Jolley 2002, Park 2002, Chiavaro et al. 2001) and from all food-contaminating aflatoxins, AFB$_1$ is usually the predominant mycotoxin (Jolly et al. 2006). Commonly, aflatoxins contaminate those foodstuffs with a high proportion of carbohydrates and lipids (Nilufer and Boyacioglu 2002) such as nuts (peanuts, pistachios, walnuts), dried fruits (figs), corn (maize), spices (pepper), seeds, milk, cocoa, beer (Cavaliere et al. 2007, Bourais et al. 2006, Brera et al. 2006, Nilufer and Boyacioglu 2002, Stroka et al. 2000).

Aflatoxins have received greater attention than any other mycotoxin because of their acute toxicological effects in humans and animals (Maroto et al. 2005). The International Agency for Research on Cancer (IARC) has classified aflatoxin as a human carcinogen group 1 (Cavaliere et al. 2007, Ali et al. 2005, IARC 2002, Chiavaro et al. 2001). Moreover, these mycotoxins are immunosuppressant, mutagenic, teratogenic and carcinogenic compounds to most organisms (Decastelli et al. 2007, Chiavaro et al. 2001). Human and animal liver is the main target organ of toxicity and carcinogenicity (Giray et al. 2007; Aycicek et al. 2005). The order of toxicity of aflatoxins, AFB$_1$> AFG$_1$> AFB$_2$> AFG$_2$ seems to stem from its structure; the portion of AFB1 terminal furan is the critical point for determining the degree of biological activity of this group of mycotoxins (Bourais et al. 2006, Chiavaro et al. 2001).

Human exposure to aflatoxins occurs primarily through intake of contaminated food, although there is evidence that inhalation of these toxins can also happen due to occupational or work-related exposure (Delmulle et al. 2006, Ramos-Catharino et al. 2005). Thus, aflatoxins constitute a serious threat to human health, especially infants and children. Because infant foods prepared from formulations based on cereals are an important source of nutrition in the diet of infants and young children and are frequently the first solid food used in infant feeding (Lawrie 1998). Consequently, aflatoxin exposure seems inevitable since multiple ingredients are present in infant cereals and baby foods (cocoa, dehydrated fruits, honey, milk- or soy-based powdered infant formula mixed with different types of cereal). In addition, it is well known that the susceptibility of infants and young children to the adverse effects of mycotoxins is greater than in adults (Baydar et al. 2007, Lombaert et al. 2003).

Consequently, there is a clear need to monitor these toxic natural contaminants in foods with special attention to this critical target group and to estimate its theoretical dietary aflatoxin intake. For this purpose, the high performance liquid chromatography with fluorescence detection, analytical method most commonly used for determination of aflatoxins in foods, is useful. However, due to its weak native fluorescence, it is also necessary to use a pre or post column derivatization (Fu et al. 2008, Sforza et al. 2006).

Accordingly, current legislation has a restrictive approach; so the Commission Regulation (EC) No. 2004/683 of 13 April 2004 lays down that the content of AFB_1 for baby foods and foods prepared from cereals for infants and young children should be kept below 0.10 μg kg^{-1} (EC 2004).

International expert committees have not deemed advisable to express a tolerable aflatoxin daily intake in numerical form due to its mutagenic and carcinogenic potential. Thereby, they recommend keeping aflatoxins levels as low as reasonably possible and indicating that even a dietary exposure as little as 1 ng kg^{-1} body weight per day could contribute to a risk of liver cancer (Leblanc et al. 2005).

Therefore, owing to all the above-mentioned aspects, the main aims of this study is to determine the aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFtotal) content in most of infant cereals marketed in Spain (n = 91 from 10 different manufacturers), to evaluate the levels of these contaminants in terms of different types and production method of cereal (organic or conventional), and also, to assess the daily dietary intake of this toxic contaminants by infants fed on infant cereals for each stage of life.

Materials and methods

Infant cereal samples

A total of ninety-one samples of different infant cereals marketed in Spain from eight distinct conventional (Hero baby, Milupa, Nestlé, Nutriben, Nutricia, Ordesa, Puleva and Sandoz-Sanutri) and two organic (Biocrecimiento and El Granero Integral) manufacturers were analyzed. Infant cereal samples were provided for free by the household or purchased in pharmacies and specialised organic feeding shops from Pamplona (Navarra, Spain).

The infant cereals studied were either organic (n = 17) or conventional (n = 74) cereal based types. Besides different types of infant cereals were classified, according to the varieties used throughout the different stages of growth in a progressively diversified diet for infants aged from four months of age, in: Gluten-free based infant cereals (n = 23, specialised formula which are normally based in rice and maize which are specially designed for infants from 4 months to 6 months of age), infant cereals with fruits (n = 5, formula composed of with- or without-gluten based cereals and dehydrated fruits added, designed for infants from 4 to 6 months age old), infant cereals with milk (n = 15, formula containing follow-up infant formula which constitutes the principal liquid source of nourishment for infants aged from six months of age), infant multicereals (product formulated to satisfy needs of infants from 5 to 12 months old, based on mixed gluten cereals), infant cereals with honey (n = 16, formula similar to multicereal with addition of honey but specially designed for infants from 6 months to 2 years age old), and infant cereals with cocoa (n = 6, formula based on multicereal with cocoa added which are normally recommended for infants from 1 to 2 years age old).

These infant feeding product is usually marketed in a cardboard box of 300 g or 600 g, which contains powdered infant cereal packaged in a foil pouch. Infant cereals were preserved in their original packaging prior analysis.

Chemicals and Reagents

Aflatoxin standard (Aflastandard, R-Biopharm, Spain), a commercial solution of AFB_1, AFB_2, AFG_1 y AFG_2 in methanol, 1000 ng mL^{-1} (250 ng mL^{-1} AFB_1, 250 ng mL^{-1} AFB_2, 250 ng mL^{-1} AFG_1, 250 ng mL^{-1} AFG_2) was performed the standard calibration curves.

Methanol and acetonitrile HPLC gradient grade (Merck, Barcelona, Spain), ultrapure deionised water Type I reagent grade (Wasserlab, Noain, Spain), nitric acid 65% (Merck) and potassium bromide (Merck) were used in the preparation of the standards and the mobile phase.

Sodium chloride (Merck) and PBS (phosphate buffered solution pH = 7.4) containing potassium chloride (Panreac, Barcelona, Spain), sodium phosphate dibasic anhydrous (Panreac), potassium phosphate monobasic (Panreac) were used in the extraction and purification of aflatoxins.

The reference materials Animal feed R-Biopharm (Darmstadt, Germany) P64-ASF3 and P64-ASF4 with different contamination levels, Ground corn R-Biopharm P64-A227 and Animal feed based on cereals from FAPAS® was used for verification and validation of the analytical methodology.

Instrumental

A HPLC system 1100 Series (Agilent Technologies, Barcelona, Spain) equipped with a quaternary pump (G1311A), auto-injector (G1313A) and a fluorescence detector FP-2020 Plus (Jasco, Madrid, Spain) communicated by means of the interface LC-Net II / ADC (Jasco). Kobra Cell System (R-Biopharm) was used for post-column derivatization. Separation was carried out on a column Luna C18 (2), 4,6 x 150 mm, 5 μm particle size, 100A (Phenomenex, Torrance, United States of America) protected by a precolumn (Phenomenex): holder of precolumn, analytical guard cartridge system, 4.6 x 10 mm, cartridge guard column, C18 Cartridges Security Guard 4 x 3 mm.

Analytical procedure

Extraction and purification

Infant cereal sample (50 g) were carefully weighed and mixed with 4 g of sodium chloride and 250 mL of extracting agent ACN:H_2O (60:40) into the blender jar. After crushing and mixing for 2 min at high speed, the extract was filter through Whatman nº 4 filter paper (Whatman International Ltd. Maidstone, United Kingdom); a 25 mL volume of filtrate was evaporated (Buchi R-3000 Rotavapor Büchi Labortechnik AG, Postfach, Switzerland) for 8 minutes at a temperature of 30 °C and a rotation speed of 65 rpm. The evaporation residue was transferred into 50 mL volumetric flask and swept up with 500 μL of ACN and a solution of PBS. Finally, 10 mL of the reconstituted extract were passed through the immunoaffinity column (Aflaprep, R-Biopharm) at a flow rate of 2 mL min^{-1}. The column was washed with 2 portions of 10 mL of ultrapure water with a flow of 5 mL min^{-1}, and the aflatoxins were slowly released from the antibody using 1 mL of methanol and eluted with 1 mL ultrapure water. Finally, the eluted sample was filtered by a PVDF syringe filter (13 mm 0.22 μm Tecnokroma, Barcelona, Spain), and collected in a vial for HPLC analysis.

HPLC determination of aflatoxins

The chromatographic conditions previously optimized and validated for determination of aflatoxins in sample extract are listed in Table 1. The analysis of samples by chromatography was performed at a room temperature kept at 22 °C, using a daily filtered mobile phase and degassed by sonication.

[Insert table 1 about here]

Validation of HPLC-FLD method for aflotoxins analysis in infant cereals

The validation for HPLC-FLD detection of aflatoxins in infant cereals samples were made in accordance with the AEFI (2001) and Eurachem (1998) guides. The analytical methodology was validated in terms of linearity, selectivity, accuracy, precision and limits of detection (LOD) and quantification (LOQ).

Linearity

The linearity of the method was evaluated through the response of aqueous standards prepared in the laboratory from a multi-aflatoxins stock solution of certified concentration for each of the aflatoxins tested. Two calibration curves, with 10 standards for each aflatoxin, were prepared in two different ranges of concentration: 0.01-0.84 ng mL^{-1} and 0.8-42.4 ng mL^{-1}.

Limits of detection and quantification

The theoretical limits of detection and quantification are obtained by analyzing a series of blank matrices and applying the equations (1) and (2), respectively:

$$LOD = \frac{Y_{blank} + (3 \times S_{blank})}{b \times \sqrt{n}} \quad [1]$$

$$LOQ = \frac{Y_{blank} + (10 \times S_{blank})}{b \times \sqrt{n}} \quad [2]$$

where *n* is the number of replicas, *b* slope of the calibration curve, Y_{blank} instrumental signal mean and S_{blank} its standard deviation. However, these values should be checked for routine use by means of the experimental method of Eurachem. It consists in preparing sample series of decreasing concentration of analyte, which were evaluated for six consecutive times and finally representing the RSD (%) of the accuracy versus the concentration of each sample tested. Usually, a fixed criterion RSD = 20 % for the detection limit and RSD = 10 % for the limit of quantification was used.

Selectivity

Selectivity was tested by comparing the chromatograms of blank samples, standards and spiked naturally contaminated infant cereals samples at different concentration levels.

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Accuracy and Precision

Accuracy and precision were evaluated through the analysis of spiked samples and three reference materials, injected in triplicate, for several days. Also, an interlaboratory FAPAS test was done for the analysis of both, isolated and total aflatoxin content in animal feed sample.

Statistical analysis

All statistical analyses were run using SPSS program version 15.0.1. The Kolmogorov-Smirnov statistic was used to assess whether the data set were normally distributed. Different groups of infant cereals samples (cereal production methods, type of cereal, predominant cereal and infant cereals manufacturers) were compared through non-parametric Kruskal-Wallis test and a Mann-Whitney U-test with a statistical significance set at $p < 0.05$. Samples below the LOD and found detected signal were assigned a value of (LOD)/2 for calculation of the statistical parameters of distribution characterization.

Results and discussion

Assay validation

The retention times studied in naturally contaminated samples and standards containing aflatoxins (AFG_2, AFG_1, AFB_2 and AFB_1) were: 8.2, 10.6, 12.9 and 16.8 minutes, respectively (Figure 1).

[Insert figure 1 about here]

Selectivity

A useful analytical method should permit resolution and detection of the analytes of interests from others interfering toxins and co-eluting sample compounds. Possible interferences were evaluated by analyzing non contaminated samples and by spiking naturally contaminated samples at different levels of concentration. No interfering peaks was observed and no significant peaks were found at retention times of analytes in blank samples. Figure 1 shows the chromatograms of separation of blank sample (a), of a standard calibration (2 ng mL^{-1}) (b) and from a infant cereal naturally contaminated. The aflatoxins elute as sharp symmetrical peaks and the resolution was optimum whit the mobile phase composition described.

Linearity

In all regressions the requirements for linearity were met. The correlation coefficient for each calibration curve was greater than 0.999 and the RSD of response factors for each concentration assayed were below than 7%. The relative standard deviation of the slope is less than 2%, slope is significantly different from zero at 28 degrees of freedom (d.f.), ttab(28 d.f.) = 2.05, and relative standard deviation of the retention times does not exceed a 5%. The standard curves for assayed aflatoxins by HPLC were linear from 0.01-

0.84 ng mL^{-1} and 0.8-42.4 ng mL^{-1} equivalent to 0.01-0.84 µg kg^{-1} and 0.8-42.4 µg kg^{-1} of aflatoxins in infant cereal samples.

Limits of detection and quantification
The detection limits obtained were AFB$_1$: 3, AFB$_2$: 2, AFG$_1$: 2, AFG$_2$: 2 ng·kg^{-1} and quantification limits of AFB$_1$: 12; AFB$_2$: 9; AFG$_1$: 11; AFG$_2$: 10 ng·kg^{-1}.

Accuracy and Precision
Aflatoxin-free infant cereal samples were spiked with standard aflatoxins solutions at levels of 2.50, 6.25 and 12.50 µg kg^{-1}. All samples were tested as six replicates The overall recoveries of AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$ were (mean ± s.d.): 83.7 ± 3.8 %, 83.2 ± 3.2 %, 81.9 ± 2.4 % and 70.2 ± 1.1 %, respectively. As show in Table 2, recoveries and relative standard deviations (RSDr) for within-day samples at low level assayed concentration were 70.0-87.9 % and 0.54-6.66%; at intermediate level were 69.2-80.4 % and 2.73-3.86 % and at high level were 71.4-82.8 % and 3.92-7.59%, respectively. However, the values of RSDR from between-day analysis were slightly higher, 3.72-18.51 % at low level assayed concentration, 4.48-5.80 at intermediate level and 5.36-9.09 % at high level. These values fall within the reference ranges of accuracy and precision set by the report UNE-CR 13505 (2003), keeping over the whole application range.

[Insert table 2 about here]

The accuracy of the method was also demonstrated by the agreement of the results obtained for reference materials Animal feed R-Biopharm P64-ASF3 (n = 9, AFB$_1$: 5.37 ± 0.35 µg kg^{-1}) and P64-ASF4 (n = 6, AFB$_1$: 19.21 ± 1.73 µg kg^{-1}, AFB$_2$: 1.57 ± 0.06 µg kg^{-1}), and Ground corn R-Biopharm P64-A227 (n= 3, AFB$_1$: 8.00 ± 0.45 µg kg^{-1}) with the 95 % confidence interval certified values (AFB$_1$: 6.6 ± 1.6, 19.5 ± 3.6 and 9.7 ± 1.5; AFB$_2$: 1.0 ± 0.9 µg kg^{-1}, respectively).

Besides during this study, the laboratory participated in one interlaboratory proficiency testing provided by the Central Science Laboratory (CSL) with a satisfactory z-score.

Global distribution and incidence of aflatoxin in infant cereals
The preliminary statistical study of aflatoxin content in infant cereal shows the typical behaviour of other contaminants in food matrices. The distributions of aflatoxins concentration present a positive skew with scores clustered to the left at the low values (right-skewed distribution) and a positive kurtosis value indicating a rather peaked distribution (leptokurtic distribution). Besides the result of normality test does not suggest a Gaussian distribution. It allows the use of median and interquartile range (Q$_1$;Q$_3$) as most

representative parameters concerning the statistical description of data better than mean and standard deviation. Nevertheless, mean and standard deviation is also provided as additional and useful informative values.

Table 3 shows a significant incidence of total aflatoxin in the infant cereal studied, reaching a 66 % (60 samples with a aflatoxin concentration above the detection limit out of 91 infant cereals analyzed) and corresponding with a 46 %, 40 %, 34 % and 11 % for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. This fact prove the agreements found in literature (Blesa et al. 2004). Therefore, from a global point of view, it is possible to establish the highest content for AFB_1, an intermediate level for AFG_1 and lastly, a negligible values for AFB_2 and AFG_2 (Table 3).

[Insert table 3 about here]

In spite of these low global values, seven infant cereals out of the 91 formulations analyzed in this study, outstanding values as outliers in table 3 (0.12; 0.21; 0.35; 0.47; 0.94; 1.98; 3.11 µg kg^{-1}) exceeded the AFB_1 content set in the Commission Regulation (EC) 2004/683 (EC 2004).

The presence of other mycotoxins different than aflatoxins (Ocratoxin, citrinin, fumonisin, deoxynivalenol, zearalenone and alkaloids) has been previously demonstrated in infant cereal and/or breakfast cereals (Leblanc et al. 2005, Molinié et al. 2005, Biffi et al. 2004, De Castro et al. 2004, Food Standards Agency 2004, Park et al. 2004, Lombaert et al. 2003, Kim et al. 2003, Candlish et al. 2000). Thereby, aflatoxins have also been reported in based on cereal products although at not detected levels (Leblanc et al. 2005, Food Standards Agency 2004, Candlish et al. 2000, Solovey et al. 1999) with the exception of breakfast cereals: 2 µg kg^{-1} in one sample in United Kingdom (Candlish et al. 2000), <5-35 µg kg^{-1} in Egypt (El-Sayed et al. 2003), 0.05-4.30 µg kg^{-1} in Greece (Villa and Markaki, 2009) and 0.002-1.00 µg kg^{-1} in Canada (Tam et al. 2006).

Few studies of aflatoxins in infant cereals were found in the literature. In Turkey, Baydar et al. (2007) found a highlight AFB_1 level (mean: 0.89 ± 1.10 µg kg^{-1}, range: 0.10-6.04) in 55 of the 63 samples (87 %); moreover eight of the infant cereals studied exceed the allowable limits for baby food in force. Also, other study (Tam et al. 2006) carried out with Canadian infant cereals exclusively found a 1% of samples (2 out of 177) exceeding the European Union legislation. The incidence was established in 50 % and the positive ranges of concentration (AFB_1: 0.002-0.996, AFB_2: 0.002-0.136, AFG_1: 0.008-0.271, and AFG_2 0.008-0.048 µg kg^{-1}) were consistent with our findings.

Influence of cereal production method on aflatoxin content in infant cereals

It is well documented that mycotoxin are mainly brought in food chain through two known ways: direct contamination in consequence of fungi growth, and indirect as a result of use in manufacture of any ingredient previously contaminated (Wood et al. 2001).

In this sense, there is considerable controversy surrounding mycotoxins regarding the safety of organic and conventional products. The study in detail of literature do not make clear a lower contamination rate of mycotoxin in cereal based foods ecologically produced (Magkos et al. 2006), since some studies (Biffi et al. 2004; Malmauret et al. 2002, Birzele et al. 2000) show both contradictory and discrepant incidence values for different types of cereal products organically or conventionally obtained (Lairon 2009, Biffi et al. 2004, Birzele et al. 2000). In this respect, Brera et al. (2006) reported a considerable level of concentration for AFB_1 and AFB_2 in organic corn samples (<0.15-25.78 and <0.15-1.96 µg kg^{-1}, respectively) in comparison with conventional ones (<0.15-5.06 and <0.15-0.67 µg kg^{-1}, respectively), and exclusively AFG_1 (<0.15-1.85 µg kg^{-1}) in ecologically produced samples.

In this study, lower aflatoxin values in infant cereals based on raw materials produced in a conventional way (AFB_1: <LOD (n.d.;0.02), AFB_2: n.d. (n.d;0.01), AFG_1: <LOD (n.d.;0.004), AFG_2: n.d. (n.d.;<LOD) and total AF: 0.01 (<LOD;0.04 µg kg^{-1})) in comparison with those infant cereal organically or ecologically produced (AFB_1: 0.02 (0.02;0.21), AFB_2: n.d. (n.d;0.03), AFG_1: 0.02 (0.01;0.05), AFG_2: 0.007 (n.d.;0.02) and total AF: 0.05 (0.03;0.31 µg kg^{-1})), are readily apparent. Apart from AFB_2, this fact was confirmed with the high statistical significance criterion found when are compared by means of Mann-Whitney U-test ($p < 0.001$). In this respect, the box plots are useful to visualize the differences observed in both infant cereals distributions (Figure 2).

[Insert figure 2 about here]

In the light of these findings, it seems appropriate to argue that the mode of production of cereals is not linked with an additional criterion of food safety regarding to aflatoxin content. Nothing else but the lack of synthetic fungicides or chemical agents, not allowed in ecological production, turn the organic crops into more susceptible to fungi contamination, resulting in a increased risk of mycotoxin contamination (Lairon 2009, Pussemier et al. 2006).

Aflatoxins content in the different types of infant cereal

Table 4 summarizes the aflatoxin B_1, B_2, G_1, G_2 and total content in the different types of infant cereal studied, classified according to the non- or organic origin of cereal used for manufacture and the regimen feeding during the first stages of life. Undoubtedly, the differences in all aflatoxins contents provided by

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different commercial infant cereals analyzed are of special relevance. Therefore, it is possible to establish the main source of aflatoxin contamination in relation to the type of infant cereal.

[Insert table 4 about here]

The highest aflatoxins levels were provided by cereals with cocoa. By way of illustration, independently of production method, both AFB_1 (ecological: 3.11 µg kg^{-1}, conventional: 0.07 (n.d.;0.08) µg kg^{-1}) and AFG_1 (ecological: 0.42 µg kg^{-1}, conventional: 0.02 (0.01;0.02) µg kg^{-1}), and in a lesser extend AFB_2 (ecological: 0.41 µg kg^{-1}, conventional: 0.01 (n.d.;0.02) µg kg^{-1}) and AFG_2 (ecological: 0.07 µg kg^{-1}, conventional: 0.005 (<LOD;0.011) µg kg^{-1}), levels are highlighted. In this respect, it is understandable that cocoa represent an important contamination source rather than the intrinsic content provided by the cereal as such, owing to the more susceptible to fungal attack during the harvest, drying, storage or processing of cocoa bean. Different studies have revealed high contents of aflatoxins in this food and derivatives (Kumagai et al. 2008, Aycicek et al. 2005). Likewise, it is worth mentioning the high levels of aflatoxin B_1 supplied by gluten-free cereals (ecological: 0.02 (0.01;0.25), conventional: 0.01 (n.d.;0.02)) and cereals with fruits (ecological: 0.11 (n.d.;0.21) and conventional: 0.005 (n.d.;0.014)), due to early incorporation and important nutrition source of this kind of formulations into infant diet during the first months of life. These results agree with those of other authors who pointed that the raw materials used in these products (rice, corn and dried fruits) are risks factors of aflatoxin contamination (Kumagai et al. 2008, Lipigorngoson et al. 2003, Shenasi et al. 2002, Tabata et al. 1993).

On the other hand, cereals with milk (conventional: n.d. (n.d.;< LOD) µg kg^{-1}), cereals with honey (ecological: 0.02, conventional: <LOD (n.d.;<LOD) µg kg^{-1}) and multicereals (ecological: 0.02 (0.02;0.07) µg kg^{-1}, conventional: <LOD (n.d.; <LOD) µg kg^{-1}) are infant cereals that contain lowest levels of aflatoxin B_1, in contrast to the high values expressed by Baydar et al. (2007) in milk-based infant food (0.73 ± 1.11 µg kg^{-1}), cereal (0.80 ± 0.44 µg kg^{-1}) and cereals with milk (1.93 ± 2.08 µg kg^{-1}). Moreover, it is not surprising that multicereal and cereal with milk or honey, are comparable and rather poor in AFB_2 and AFG_2.

Aflatoxins were detected in all types of infant cereals studied, in both non- and organic formulations which two and five infant cereals, respectively, overpassed the European maximum level allowed for AFB_1, particularly, three gluten-free cereals, two cereals with dehydrated fruits, one cereal with cacao and one multicereal. In this sense, the different mixed cereals used in formulation as well as the added ingredients might bring about a increase of aflatoxin concentration, calling for routine analysis of final product and raw materials to kept aflatoxin levels as low as possible in the infant cereals.

11

Aflatoxin content and infant cereal manufacturers

Box diagrams expressed in Figure 3 show the concentrations of aflatoxins determined in the different infant cereals belonging to the ten manufacturers (conventional: companies 1-8, organic: companies 9 and 10) which provide the most cereal infant sold in Spain. Globally, the meticulous care shown by manufacturers 1-7 and 9 is curious, while manufacturers 8 and 10 show quite the opposite with a large range of aflatoxin concentration, including all the formulations outside of the EC legislation (two and five infant cereals, respectively). It is also necessary to mention manufacturer 2 and in a certain distance behind manufacturer 4, which include numerous formulations in their stocks with a discrete aflatoxin contribution from prepared infant cereals.

[Insert figure 3 about here]

In view of these results and moves to reduce the level of aflatoxin in infant cereals, it seems suitable to call for an effort to control as far as possible the critical points of aflatoxin contamination from main ingredients and to analyse frequently or routinely the aflatoxin contents, especially in those formulations from manufacturers using raw materials of potential impact. At this respect, cocoa based infant cereals are a case of special relevance which are usually formulated to contain cocoa at 10 % and 5-8 % in organic and conventional infant cereals, respectively. If this ingredient is considered as the main aflatoxin contributor by itself, the estimated AFB_1 content in this raw material used by organic and conventional manufacturers might reach a high level around 31.1 $\mu g\ kg^{-1}$ and 0.82-1.32 $\mu g\ kg^{-1}$, respectively. Unfortunately, cocoa is not included in the Commission Regulation (EC) 1881/2006 as specified foodstuff (EC 2006). Therefore, tighten control practices on this ingredient should be demanded.

Estimated daily dietary aflatoxins intake

Infant cereal certainly is the traditional choice of first solid food for infant fed on. It is often mixed with infant formula or breast milk until it has a slurry consistency. Commercially prepared infant cereals are convenient for the first-stage of growth. But also consequently potential vehicle for toxins through the infant feeding. Hence, the estimation of theoretical dietary intake of afaltoxins by infants fed on studied cereals is of particular relevance.

Daily dietary intake was calculated using the aflatoxin concentration median value determined in the different types of infant cereals according to feeding tables and recommended doses by manufacturers for the different stages of infancy (4 month: 48 g, 5 month: 60 g, 6 month: 72 g, 7 - 12 month 84 g, 13 -

24 month: 96 g) and its mean body weight (4 month: 6.5 kg, 5 month: 7.25 kg, 6 month: 7.75 kg, 7 - 12 month 9 kg, 13 - 24 month: 13 kg).

Figure 4 shows the daily intake for the total and each aflatoxin studied supplied by non- and organic infant cereals depending on the different stages of growth. Upon the whole, as expected, daily intake values provided by ecological infant cereals ranging 0.12-29.06, 0.02-3.82, 0.15-3.91, 0.05-0.68 and 0.17-37.47 ng kg^{-1} body weight per day for AFB_1, AFB_2, AFG_1, AFG_2 and AFtotal, respectively, were far greater than those supplied by conventional infant cereals (AFB_1, 0.01-0.62; AFB_2, 0.02-0.13, AFG_1, 0.005-0.156; AFG_2 0.0015-0.05 and AFtotal 0.08-0.94 ng kg^{-1} b.w.). However, apart from organically produced infant cereals with cocoa which involve an inadmissible risk for infant health, the rest of infant cereals are below 1 ng kg^{-1} body weight per day. Although, it is also appreciated that the infant formulations used during the first stages of beikost (gluten-free cereals and cereals with dehydrated fruits) provide a higher aflatoxin exposition than other formulations (multicereals, cereals with milk or honey) subsequently used (Figure 4).

[Insert figure 4 about here]

Fortunately, exclusively cocoa based cereals analyzed are close to the high level of aflatoxin contamination reported in Turkish infant formulae or corn based diets for infants in India, 24.16 and 47 ng kg^{-1} b.w. of AFB_1, respectively (Baydar et al. 2007, Vasanthi and Bhat, 1997). The implementation and application of European Union legislation has largely been responsible to improve the safety of the ingredient supply chain. The directives on contaminants is part of the legislative push to reduce levels of mycotoxins in the crops and to minimise their formation in stored grain. This fact has been proved by the significant reduction in daily exposure of European infants to aflatoxin (0.32 ng kg^{-1} b.w.) during the last decade in comparison with those values provided in the nineties (2.4-4.5 ng kg^{-1} b.w.), leading to a decrease of the risk infant population to aflatoxin up to 3.4 % (Leblanc et al. 2005, Verger et al. 1999). Likewise, the infant risk for aflatoxin could be assessed by means of a comparison with the little information in literature concerning daily intake from total diet studies and nutrition surveys. A lower intake values of 0.15 ng kg^{-1} b.w. in Australia (Australian Market Basket Survey, 1992), 0.80 ng kg^{-1} b.w. in Sweden (Thuvander et al. 2001), 0.26 ng kg^{-1} b.w. in several members of the European Community (Park et al. 2004) from standard diets similar to our findings have been established while the monopolised diet by rice, for example 296 ng kg^{-1} b.w. in Vietnam (Nguyen et al. 2007) represent an important risk of cancer.

Conclusion

The obtained results suggest that newborns fed on infant cereals studied are exposed to low levels of aflatoxin excluding those formulations which fail to comply with European Regulation. Nevertheless aflatoxin contamination of foods including infant cereals is evident, and in this context, due to its special role in infant nutrition, a more rigorous and good quality control of those ingredients used in infant cereal manufacture with higher health hazard should be exercised. Moreover, governmental strategies driven forward to carry out a more careful selection of raw materials used by infant food manufactures should be also taken into consideration.

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Table 1. Chromatographic conditions for aflatoxins determination in infant cereal.

Parameters	HPLC conditions	Parameters	Kobra Cell conditions
Injection volume (μL)	100	Current (μA)	100
Temperature	22 °C	Temperature	22 °C
Flow	Isocratic	Flow	Uniform
Mobile phase (v/v)	Water:ACN:MeOH 60:35:5 119 mg KBr 98μL HNO$_3$	Reaction tube length (cm)	34
Caudal (mL/min)	1.0	Caudal (mL/min)	1.0
λ_{ex} (nm)	362	Internal diameter teflon tube (mm)	1.58
λ_{em} (nm)	455	Need for additional devices	No
Gain	100	Location	Between the column and the detector

Table 2. Precision and accuracy of the method for aflatoxins determination (n = 15).

	Assayed concentration								
	2.50 µg kg⁻¹			6.25 µg kg⁻¹			12.50 µg kg⁻¹		
	Recovery	RSD_r	RSD_R	*Recovery*	RSD_r	RSD_R	*Recovery*	RSD_r	RSD_R
AFB_1	87.9±4.8	0.54	5.61	80.4±4.3	3.86	5.37	82.8±7.5	3.92	9.09
AFB_2	85.7±5.2	3.11	6.05	79.2±3.5	3.12	4.48	82.4±6.2	4.86	7.35
AFG_1	84.0±3.2	1.52	3.72	79.2±4.5	2.73	5.65	82.4±7.4	7.59	9.05
AFG_2	70.0±4.8	6.66	18.51	69.2±2.6	3.17	5.80	71.4±3.8	5.21	5.36

Table 3. Incidence and statisticians global distribution of the different aflatoxins.

Aflatoxin	n	Non detected	Detected		Median ($\mu g \cdot kg^{-1}$)	Mean ± s.d. ($\mu g \cdot kg^{-1}$)	(Q1;Q3) ($\mu g \cdot kg^{-1}$)	Outliers
		n.d. (%)	<LOD (%)	>LOD (%)				
AFB$_1$	91	37	17	46	< LOD	0.09±0.40	(n.d.;0.02)	0.12, 0.21, 0.35, 0.47, 0.94, 1.98,3.11
AFB$_2$	91	60	0	40	n.d.	0.01±0.05	(n.d.;0.01)	0.05,0.05,0.09,0.14,0.41
AFG$_1$	91	34	32	34	< LOD	0.02±0.06	(n.d.;0.02)	0.07,0.17,0.30,0.42
AFG$_2$	91	57	32	11	n.d.	0.004±0.012	(n.d.;0.002)	0.01,0.01,0.02, ,0.02,0.02,0.03, 0.06,0.06,0.07
Total	91	19	15	66	0.02	0.12±0.51	(0.01;0.04)	0.31,0.40,0.62,1.31,2.43,4.02

Table 4. Aflatoxins content in types of infant cereal (µg kg^{-1}).

Analyte	Type	Production	n	Mean	Std. dev.	Median	(Q₁,Q₃)
AFB₁	Cocoa	Conventional	6	0.05	0.04	0.07	(n.d.,0.08)
		Ecological	1	3.11			
	Fruits	Conventional	5	0.03	0.05	0.005	(n.d.,0.01)
		Ecological	2	0.11	0.15	0.11	(n.d.,0.21)
	Honey	Conventional	16	0.004	0.01	< LOD	(n.d.,< LOD)
		Ecological	1	0.02			
	Milk	Conventional	5	< LOD	0.004	n.d.	(n.d.,< LOD)
		Ecological	-				
	Multicereal	Conventional	19	0.004	0.01	< LOD	(n.d.,< LOD)
		Ecological	6	0.18	0.37	0.02	(0.02,0.07)
	Gluten-free	Conventional	23	0.03	0.07	0.01	(n.d.,0.02)
		Ecological	7	0.36	0.74	0.02	(0.01,0.25)
	Overall	Conventional	74	0.02	0.04	< LOD	(n.d.,0.02)
		Ecological	17	0.41	0.86	0.02	(0.02,0.21)
AFB₂	Cocoa	Conventional	6	0.01	0.01	0.01	(n.d.,0.02)
		Ecological	1	0.41			
	Fruits	Conventional	5	0.01	0.01	0.01	(n.d.,0.01)
		Ecological	2	0.01	0.02	0.01	(n.d.,0.03)
	Honey	Conventional	16	0.01	0.01	0.005	(n.d.,0.01)
		Ecological	1	n.d.			
	Milk	Conventional	5	0.01	0.01	0.01	(n.d.,0.01)
		Ecological	-				
	Multicereal	Conventional	19	0.003	0.01	n.d.	(n.d.;n.d)
		Ecological	6	0.03	0.06	0.01	(n.d.,0.01)
	Gluten-free	Conventional	23	0.01	0.01	n.d.	(n.d.,0.005)
		Ecological	7	0.02	0.04	n.d.	(n.d.,0.02)
	Overall	Conventional	74	0.01	0.01	n.d.	(n.d.,0.01)
		Ecological	17	0.04	0.10	n.d.	(n.d.,0.03)
AFG₁	Cocoa	Conventional	6	0.02	0.01	0.02	(0.01,0.02)
		Ecological	1	0.42			
	Fruits	Conventional	5	0.003	0.01	< LOD	(n.d.,< LOD)
		Ecological	2	0.01	0.02	0.01	(n.d.,0.03)
	Honey	Conventional	16	0.003	0.005	< LOD	(< LOD,< LOD)
		Ecological	1	0.02			
	Milk	Conventional	5	0.01	0.02	< LOD	(< LOD,< LOD)
		Ecological	-				
	Multicereal	Conventional	19	0.004	0.01	< LOD	(n.d.,0.01)
		Ecological	6	0.04	0.06	0.02	(0.01,0.02)
	Gluten-free	Conventional	23	0.003	0.01	n.d.	(n.d.,< LOD)
		Ecological	7	0.06	0.11	0.02	(0.01,0.05)
	Overall	Conventional	74	0.005	0.01	< LOD	(n.d.,0.004)
		Ecological	17	0.07	0.12	0.02	(0.01,0.05)
AFG₂	Cocoa	Conventional	6	0.01	0.01	0.005	(< LOD,0.01)
		Ecological	1	0.07			
	Fruits	Conventional	5	< LOD	0.003	n.d.	(n.d.,< LOD)
		Ecological	2	0.01	0.01	0.01	(n.d.,0.02)
	Honey	Conventional	16	< LOD	0.001	n.d.	(n.d.,< LOD)
		Ecological	1	0.01			
	Milk	Conventional	5	< LOD	0.01	< LOD	(n.d.,n.d.)
		Ecological	-				
	Multicereal	Conventional	19	< LOD	0.003	n.d.	(< LOD,< LOD)
		Ecological	6	0.01	0.02	0.01	(n.d.,0.01)

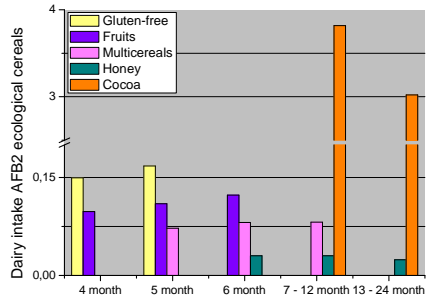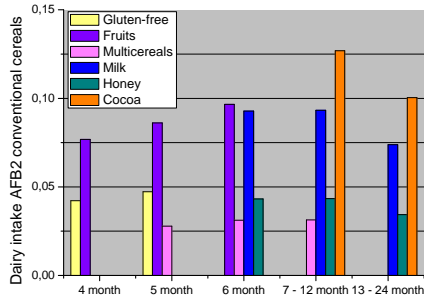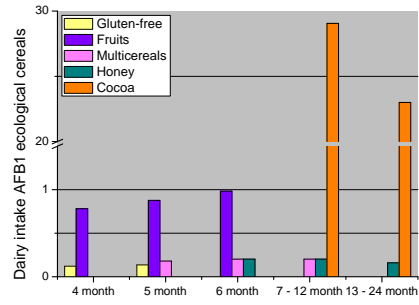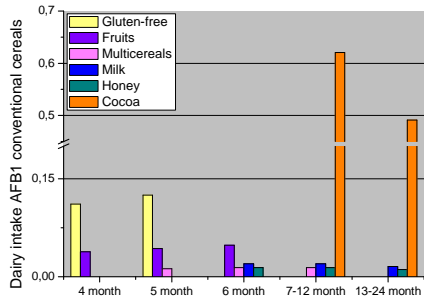|						
Gluten-free	Conventional	23	< LOD	0.002	n.d.	(n.d.,n.d.)
	Ecological	7	0.02	0.02	0.01	(n.d.,0.02)
Overall	Conventional	74	< LOD	0.003	n.d.	(n.d, < LOD)
	Ecological	17	0.02	0.02	0.01	(n.d.,0.02)
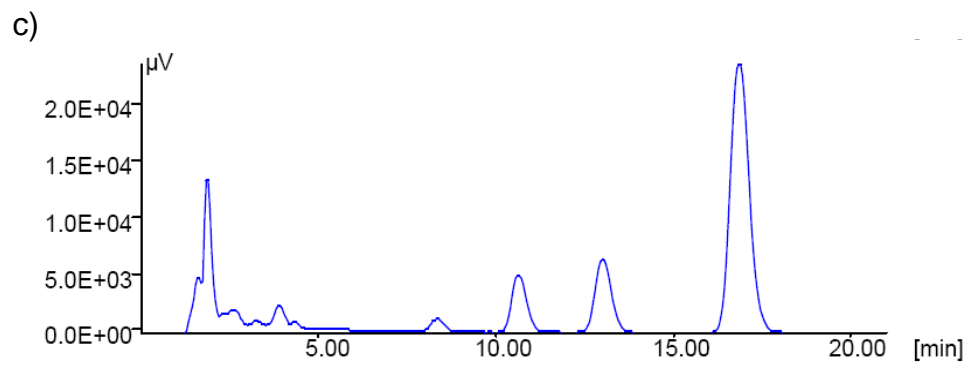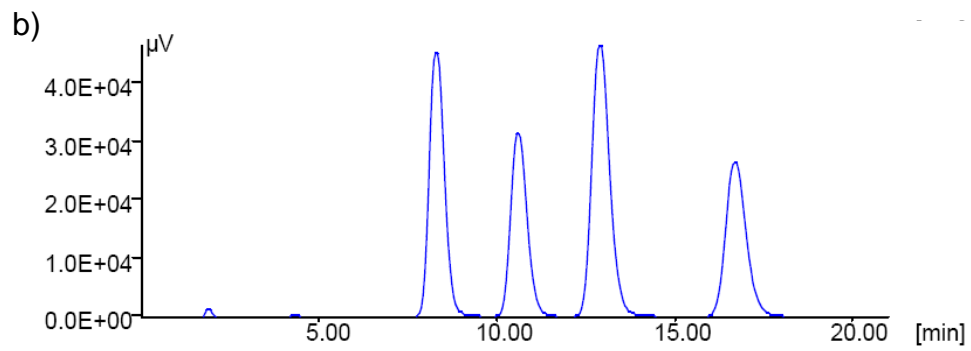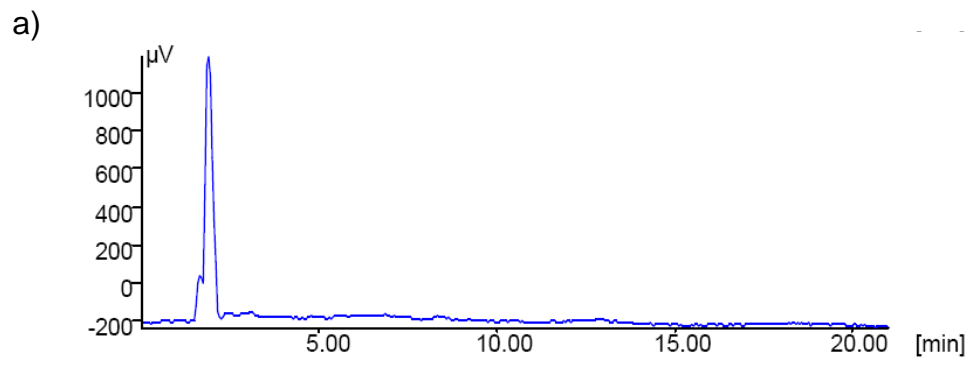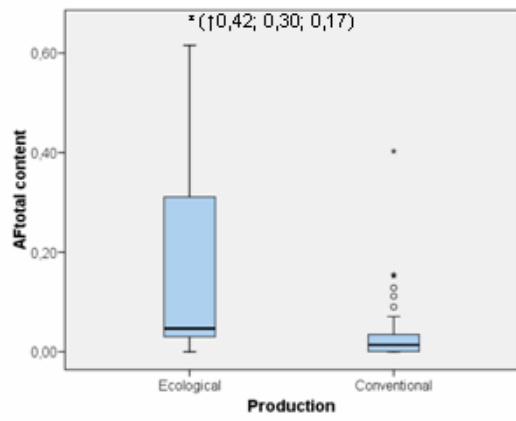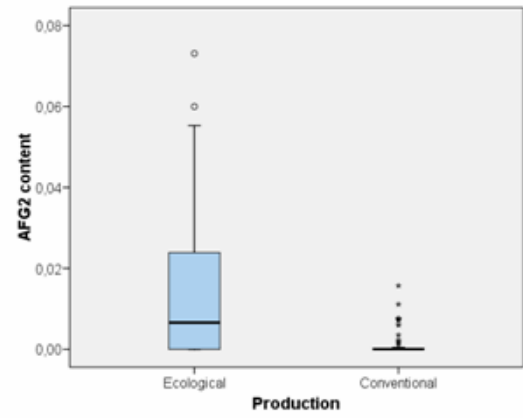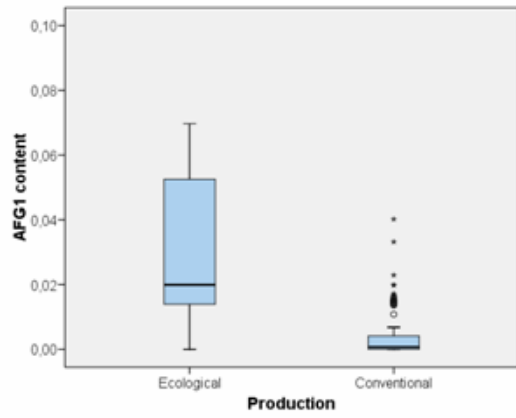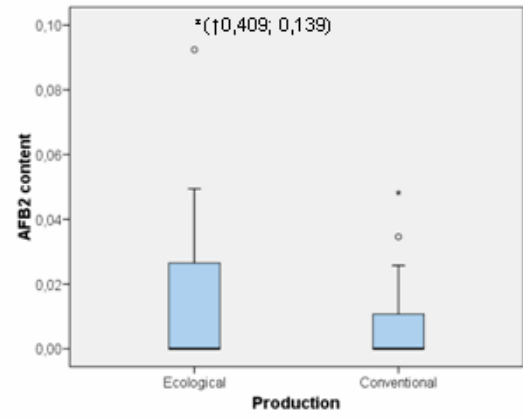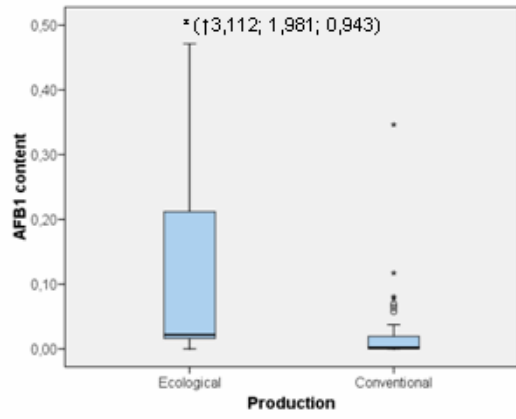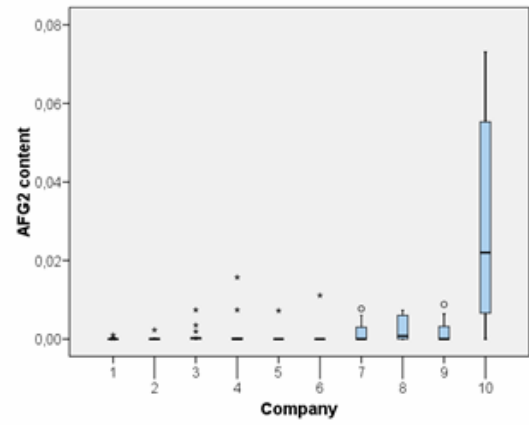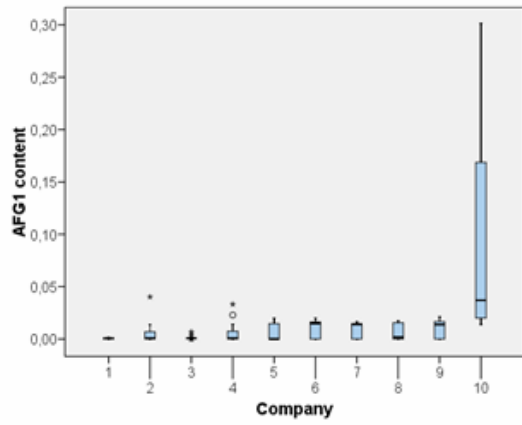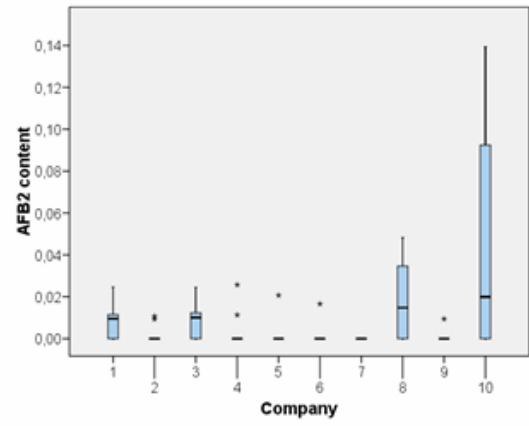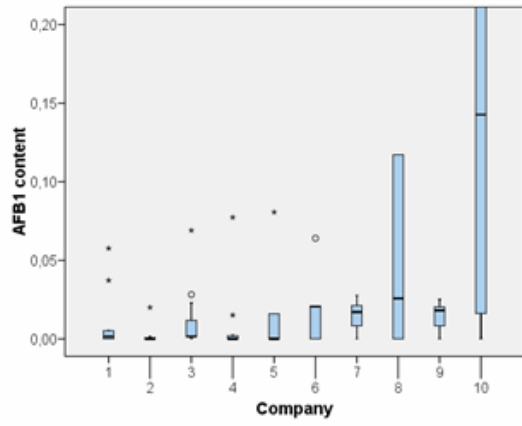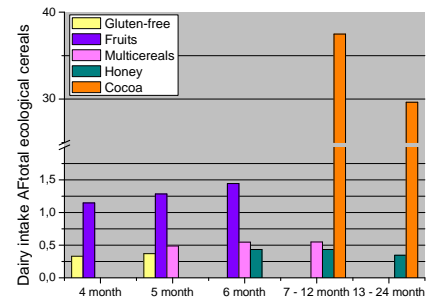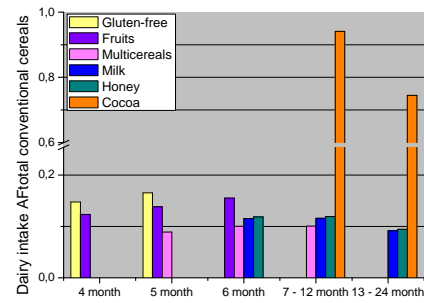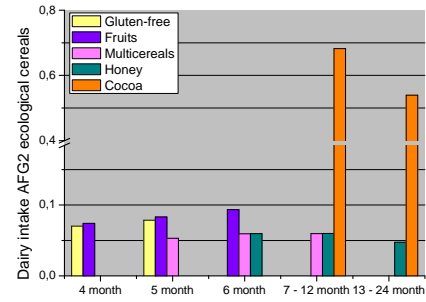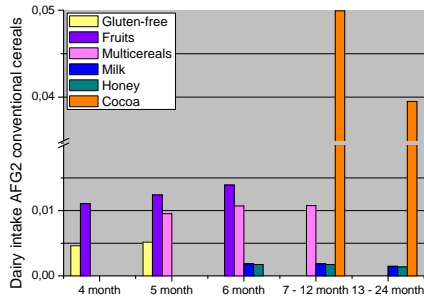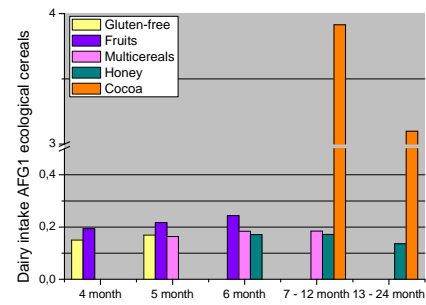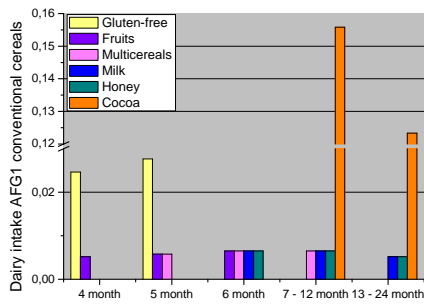
Figure legends

Figure 1. a) Chromatogram of a blank sample, b) Chromatogram of a standard calibration curve (2ng mL^{-1}, c) Chromatogram of a natural contaminated sample of infant cereal (Elution order AFG$_2$, AFG$_1$, AFB$_2$ and AFB$_1$).

Figure 2. Aflatoxins content in non- and organic infant cereals (μg kg^{-1}).

Figure 3. Aflatoxins distributions in infant cereals provided by different manufacturers (μg kg^{-1}).

Figure 4. Daily dietary aflatoxins intake provided by infant cereals (ng kg^{-1} body weight per day).