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Mycotoxin Contamination of Food in Ecuador*

A: Aflatoxins

Key words: Aflatoxin, ELISA, Ecuador, Risk assessment

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Introduction

The contamination of foods and feeds with biocontaminants is a worldwide problem which may present a hazard to human and animal health (1). Within the mycotoxins, aflatoxins (especially AFB_1) are of major concern; they were classified as probable human carcinogens (2) since there is sufficient epidemiologic evidence to link the consumption of food containing aflatoxin with the occurrence of primary liver cancer (PLC) in humans and animals. Most of the existing regulatory limits on the occurrence of mycotoxins in foods and feeds focus on aflatoxins (3).

Aflatoxin contamination poses a serious problem to tropical and subtropical areas (4, 5). In 1991, the state of knowledge about the mycotoxin situation (matrices affected, frequency and extent of contamination) in Ecuador, South America, allowed neither basic estimations of the intake of aflatoxins nor the performance of any risk evaluation for Ecuadorians (6). This situation is explained on one hand by the lack of regulatory limits on the mycotoxin contamination in foods and feeds in Ecuador and on the other hand by the lack of finances in order to establish an efficient control system.

Ecuador (281 350 km^2, 10 million inhabitants in 1990 (7)) represents a unique variety of climates: inter-Andean tropic; subtropic; moderate; cold and glacial; coast tropical humid; tropical monsoon; tropical dry and tropical orient (8, 9). Rainfalls vary from 200 to 5000 mm/year and relative humidities vary with season, reaching in the rainy, humid winter season up to 85% in the mountain region and up to 100% in the coastal region and the east. Therefore, almost every agricultural product may be grown in the country and growth conditions for mycotoxin producing fungi are present, too. Rice is produced in the coast and in the east; hard endosperm corn in

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the coast, the east, the tropical and subtropical inter-Andean region; medium hard and soft endosperm corn are produced in the inter-Andean subtropical to cold region; beans are produced all over the country; peanuts come from the inter-Andean tropical region, the coast and the east; and milk is mainly (73%) produced in the inter-Andean tropical to cold region (10–13).

The aim of the present work was to improve the hardly existing information on aflatoxin contamination in Ecuadorian foods and feeds in order to recognise existing problems, enabling a basic risk evaluation and provide information about causes of mycotoxin contamination. The occurrence of other mycotoxins suchs as ochratoxin A, vomitoxin (DON), T-2 toxin and fumonisin in Ecuadorian foods and feeds is dealt with in the adjacent part B of this work.

Materials and methods

Chemicals

Aflatoxins B_1, B_2, G_1, G_2 and M_1 (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1) were purchased from Sigma Chemical Company (St. Louis). They were diluted, quantified and tested for chromatographic purity according to (14). Distilled or deionized water was used throughout. Reagents and solvents (with at least p.a. quality) were purchased from Merck, Romil, Fluka, Sigfried, Riedl-de Haen or Baker. Benzene and toluene (both distilled) and $K_2Cr_2O_7$ were kindly provided by Dr. Espin de Rivera from the Instituto Nacional de Investigacion Agropecuaria (INIAP), Quito. Folded filter papers used were from Schleicher & Schüll, type 597 $^1/_2$ for total aflatoxins and 595 $^1/_2$ for AFM_1.

Sampling

Samples were taken between April 1992 and October 1994 and included rice and subproducts (99), hard, medium hard and soft corn (89), beans (79), peanuts (31) and milk (192).

Mixed samples (1.2 to 3.0 kg) of rice, corn (including corresponding subproducts), beans and peanuts were randomly collected from different Ecuadorian climatic regions paying attention to variety, origin, type of storage, marketing and moisture content. Additionally, rice samples were collected from the installations of the Empresa Nacional de Almacenamiento e Commercialisacion (ENAC), from an uncompleted FAO project dealing with drying down rice with its own husk in Los Rios and from the Centro Ecuatoriano de Servicios Agricolas (CESA) in Daule. Additional corn samples were from the installations of the ENAC and from a FAO project (GCP/ECU/060/NET) dealing with quality and post harvesting losses of cereals and potatoes as well as their marketing. Additional bean samples originated from the above mentioned FAO- project and from a thesis work at the Escuela

Superior Politecnica (ESPOL) in Chimborazo dealing with quality control at different storage conditions (15).

Milk samples (0.25 to 1 l) were randomly collected from different Ecuadorian climatic regions and origins (regional and local collective consignments, small and large scale farms and single animals). After collection, samples were transported at 4–8 °C in a portable refrigerator (WEMO 12V). Transportation time varied between a few hours and several days.

Pretreatment of samples

Samples of rice, corn and beans were ground on a hammer mill at 6000 rpm and size-selected by a 0.8 mm mesh (wet samples: 3000 rpm, 1.2 mm mesh), homogenized for 3 min using an industrial Hobart mixer at velocity 2 and stored frozen at –25 °C until analysis. Peanut samples were treated to form a paste (hammer mill, 3000 rpm, 2 mm mesh) and stored frozen at –25 °C until analysis. Milk samples were analysed directly without pretreatment or stored at –25 °C for a maximum of one week until analysis.

Extraction of aflatoxins for ELISA analysis

Extractions were performed, with the exception of slight modifications, according to the instructions of the producer (Neogen Corp., Lansing, MI) supplied with the semiquantitative test kits (Agri-Screen for total aflatoxins). The same extraction procedures were used for the quantitative Veratox test kit subsequently used (Neogen Corp). Twenty five g of pretreated rice, corn and bean samples were mixed with 125 ml of 70% methanolic solution. These mixtures were shaken on a Lab-Line shaker (12 min, 260 rpm) at room temperature and filtered. Contact time between sample and extraction solvent totalled 45 min. The filtrates were applied to ELISA analysis without further purification. Fifty g of peanut paste were mixed with 250 ml 70% methanolic solution in a Polytron mixer (3 min, velocity 5) and filtered. Total contact time between sample and solvent was 5 min.

Milk samples were vigorously agitated and filtered in order to remove particles. 10 ml aliquots were centrifuged (15 min, 3000 rpm) on a Sigma 101 table centrifuge in order to separate fats and the aqueous phase. The lower aqueous phase was carefully removed and applied to ELISA analysis without further purification.

ELISA analysis

All reagents were purchased from Neogen Corp., Lansing, MI. The principal and reagents were as described by (16). Optical densities of the developed colours were measured in comparison to known concentrations of four reference standards by a Dynatech Mikroelisa System (MR 5000) with 650 nm filter and the concentrations of aflatoxins (primarily AFB_1) in ng/g in the test samples were reported.

During the period of work (1992–1995), the ELISA test kit changed in performance quality from semiquantitative screening (Agri-Sceen) to quantitative detection (Veratox) of aflatoxins. The sample preparation procedure remained the same with two exceptions: the extraction solvent was changed from 55 to 70% methanolic solution and the amount of sample recommended for analysis changed from 5 to 50 g.

The production of the Veratox test kit for AFM_1 was stopped for economic reasons (Mr. R. Felman, international sales manager from Neogen, personal communication).

Extraction of aflatoxins for thin layer chromatography (TLC) analysis

For rice, corn and beans: The extraction procedure was according to (17) with the following modifications in order to co-extract ochratoxin A. Forty g of pretreated sample were mixed for 90 sec with 100 ml methanol, 10 ml water and 1 ml 0.33 mol/l phosphoric acid using an Osterizer household mixer. After the addition of 30 ml water, mixing was continued for another 90 sec. The mixtures obtained were filtered and 70 ml of the filtrates were cleaned-up by partitioning under agitation (1 min) in the presence of 50 ml petroleum ether, 5 g KCl and 55 ml water in 250 ml separating funnels. The addition of a second portion of 1 ml 0.33 mol/l phosphoric acid is crucial for high recoveries of ochratoxin A in all matrices. The lower aqueous phases were collected and 20 ml water and 100 ml methylene chloride were added. After vigorously agitating for 2 min, the lower methylene chloride phases were collected into other 250 ml separating funnels, dried over anhydrous Na_2SO_4, quantitatively filtered and the filtrates evaporated to dryness on a rotary evaporator (Büchi RE 120) at 35–40 °C. Residues were quantitatively removed, dissolved in benzene/acetonitrile (98/2) and stored at 2–4 °C until analysis or transported to Switzerland for analysis.

Fifty g of milk were mixed with 1 ml 0.33 mol/l phosphoric acid and 50 ml acetone and shaken for 10 min at 260 rpm. Shaking was interrupted to add 100 ml methylene chloride and continued for another 10 min. The resulting emulsion was forced to separate with the help of 25 g celite 545 (heated at 700 °C in a Heraeus MR 170 E) and filtered. Ninety ml of the filtrates were evaporated on a rotary evaporator (Büchi RE120) at 35–40 °C. The oily residues were quantitatively removed, dissolved in 5 ml methylene chloride and transported to Switzerland for clean-up and analysis. 1.5 g of silica gel 60 (Merck Nr. 7754, use of high quality is crucial) were filled over glass wool into 20 ml chromatography columns (diameter 14 mm) and the methylene chloride sample solutions were slowly applied. The gels were extracted with 20 ml diethyl ether (distilled over $CaCl_2$ and filtered through 100 g/l aluminium oxide, activity 1, Camag Nr. 15081 in order to remove peroxides) and elution of AFM_1 was achieved with 15 ml trichloromethane/acetone (4:1). Extracts were evaporated on a rotary evaporator (Büchi KRvrTD 65/45) and residues were quantitatively transferred with methylene chloride. Extracts were

dried down with nitrogen, dissolved in toluene/acetonitrile (9/1) and stored at 2–4 °C until analysis.

Bidirectional TLC analysis of aflatoxins B/G and M_1

TLC analysis was performed as described by (17). Aflatoxins were detected fluorodensitometrically on a Desaga Scanner CD 60 (exitation wavelength 366 nm, emission wavelength > 420 nm). The amounts of aflatoxins were calculated by peak height and comparison with standards run in parallel. AFB_1 and AFG_1 were derivatised on the TLC foils by addition of 10 µl hexane/trifluoro-acetic acid (1:2) per toxin spot and heating between glass plates (10 min, 75 °C) in order to confirm their identity.

Artificial contamination of rice, corn and bean samples with aflatoxins

In order to determine detection limits for ELISA analysis and recoveries from each matrix, 25 g of samples which tested negative in prior ELISA analysis were spiked with the equivalents of 2.4, 3.6, 6.8 and 20.55 ng AFB_1/g sample. The solvent was evaporated for 2 h and the extraction procedure followed as described above.

For determination of recoveries from each matrix in TLC analysis, 70.5 ml of filtered extract of samples that tested negative in prior ELISA analysis, were spiked with aflatoxins B_1/G_1 and B_2/G_2 at concentrations of 6.25/1.875 ng toxin/g sample, respectively, followed by the cleanup procedure described above.

Artificial contamination of milk with aflatoxin M_1

The detection limit and recovery from milk for ELISA analysis was determined by spiking test tubes with equivalents of 0.125, 0.25 and 0.75 ng AFM_1/g sample, respectively and the solvent was evaporated to dryness under a gentle stream of nitrogen. Subsequently, appropiate amounts of milk from samples which tested negative in prior ELISA analysis were added to the tubes. The tubes were capped and vortexed for 2 min. After 1 h at room temperature (in the dark) the tubes were again vortexed for 2 min and analysis was carried out as described above. Spike concentrations of 0.0625, 0.083 and 1.66 ng AFM_1/ml sample were obtained by dilution of artificially contaminated milk with uncontaminated milk.

Determination of moisture content

The moisture content of food- and feedstuff samples was monitored either by a Dole 400 Moisture Meter (model PB-70-21) or by weighing before and after drying down of samples to constant weight.

Alkaline cooking (nixtamalisation)

The effect of nixtamalisation of corn on aflatoxin levels was tested experimentally as follows: 250 g native corn were boiled for 30 min (92 °C – boiling point) in presence of 0.25% (w/w) $Ca(OH)_2$ in 0.75 l of water under constant stirring (essential). Subsequently, the water was quantitatively removed (60 °C, 72 h) in a stove under vacuum until the masa (containing the pericarp) reached the initial weight. 25 g of dry mass were extracted with 70% methanolic solution using a polytron mixer and the slurry was purified by filtration over celite.

Statistics

Data below the detection limit were arbitrarily set to half of this value. Aflatoxin data were transformed [log(X+1)] prior to analysis to equalize variances (log-normal distribution). Statistical analysis of data was performed by analysis of variance (ANOVA) followed by Scheffe's post-hoc test. Significance level was 5%. Error bars in all figures represent ± one standard deviation of the untransformed data.

Results and discussion

Validation of isolation and detection procedures for aflatoxin

Recoveries and detection limits for aflatoxins by ELISA analysis was assessed by spiking experiments as described in Materials and methods (table 1). The detection limits were at 6.8 ng AF total/g of rice, corn and beans and at 0.125 ng AFM_1/ml of milk agreeing with the AOAC approval for the Veratox aflatoxin total test kit (licence No. 931201). Recoveries from extracts ranged between 71% (milk) and 112% (beans) for ELISA analysis compared to 82 ± 4% in rice, 77 ± 7% in corn and 82 ± 11% in beans for TLC analysis. The detection limit of TLC analysis was at 0.2 ng/g for all aflatoxins (17). For the determination of the detection limit of AFM_1 in milk, two sets of quatruplicate analysis were performed as described in Materials and methods. Mean values and standard deviations of recoveries with the corresponding detection limits were 12 ± 6% and 0.048 ng/g for the first set of analysis and 73 ± 3% and 0.018 ng/g for the second set. This obvious discrepancy was found to be due to the use of an inadequate silica gel (CU Chemie, Uetikon AG, No. 1530) for the first set of analyses. The recoveries could be increased significantly by the use of the appropriate extra pure silica gel 60 (Merck No. 7754).

The performance of ELISA analysis was controlled by analysing some samples by TLC. Data above the detection limit from ELISA analysis were compared by regression analysis with the values from TLC analysis and a significant correlation between the two methods was found (fig. 1). Aflatoxin B_1 accounted for the major part of total aflatoxins in all TLC analysed samples (between 82% in beans and

Table 1. Number of analyses with mean value and standard error for ELISA – spiking experiments

A: staple food items

spiked AFB_1 (ng/g)	rice		corn		beans	
	number	mean ± std.error	number	mean ± std.error	number	mean ± std.error
0.0	10	0.31 ± 0.1	10	0.23 ± 0.07	5	0.66 ± 0.42
2.4	4	0.65 ± 0.09	4	0.38 ± 0.05	2	0.9 ± 0.2
3.6	4	1.4 ± 0.07	4	0.35 ± 0.13	2	1.5 ± 0.2
6.8	16	5.5[a] ± 0.35	18	5.3[a] ± 0.29	9	6.3[a] ± 0.35
20.6	13	22[a] ± 0.61	13	19[a] ± 0.47	6	23[a] ± 0.44

B: milk

spiked AFM_1 (ng/ml)	milk	
	number	mean ± std.error
0.0	17	0.04 ± 0.005
0.063	3	0.07 ± 0.002
0.09	12	0.08 ± 0.007
0.125	12	0.11[a] ± 0.01
0.166	3	0.14[a] ± 0.002
0.25	20	0.19[a] ± 0.01
0.75	25	0.53[a] ± 0.01

[a] Results are statistically significantly different to zero-spike with p-values < 0.0003

95% in rice and corn). AFB_2 was only found in presence of AFB_1 and high amounts of AFB_2 were related to high amounts of AFB_1. AFG_1 was only found in the presence of AFB_1. The corresponding data are shown in table 2.

Among the 11 milk samples extracted in Ecuador for TLC control purposes, some tested positive in the ELISA analysis, but negative in TLC analysis performed using the inadequate silica gel (see above). In addition, the extraction procedure in Ecuador was performed with acetone from national production (Dercol Cia. Ltda), which might not reach the quality standard of other products. The importation of acetone is strictly forbidden due to the national effort of production and possible abuse in illegal drug extraction. For practical reasons the samples had to be stored frozen at –25 °C for 11 to 16 month before transportation to Switzerland at room temperature (2 days).

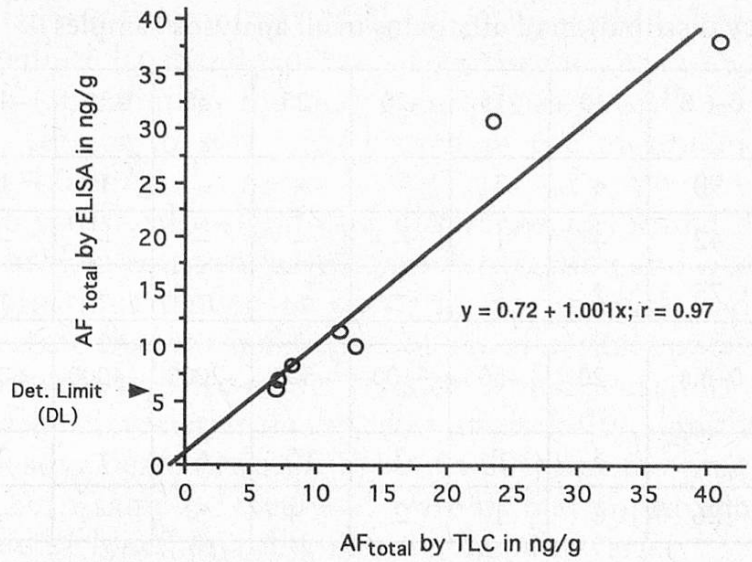

Fig. 1. Regression analysis of ELISA vs TLC from seven natural contaminated rice samples

Table 2. Number of samples and levels of contamination (in ng/g) with aflatoxins B_1, B_2, G_1 and G_2

matrix (number)	B_1			B_2			G_1			G_2			negative for AFB_1
	n	$\tilde{x}$	$\bar{x}$ (max)	*n*	$\tilde{x}$	$\bar{x}$ (max)	*n*	$\tilde{x}$	$\bar{x}$ (max)	*n*	$\tilde{x}$	$\bar{x}$ (max)	
rice (16)	14	3.0	7.2 (39)	13	0.2	0.42 (2)	5	0.0	0.23 (2)	1	0.0	0.03 (0.4)	2
corn (17)	12	0.8	175 (1426)	7	0.0	7.4 (58)	2	0.0	0.17 (1.8)	0	0.0	–	5
beans (12)	10	0.3	0.84 (7.1)	4	0.0	0.06 (0.2)	4	0.0	0.23 (2)	2	0.0	0.06 (0.4)	2

n = number of samples
$\bar{x}$ = arithmetic mean; maximal values are in parenthesis (max)~
$\tilde{x}$ = median

Occurrence of aflatoxins in different Ecuadorian foods

The frequency distributions of total aflatoxin contamination levels as determined by ELISA analysis are shown in table 3. Note that the scale is linear for rice, soft corn and beans but non-linear for peanuts, hard endosperm corn and milk. Most striking are the very high contamination levels of peanuts and hard endosperm corn, whereas moderate contamination levels were determined for rice, beans and soft endosperm corn. Many milk samples were analysed in order to trace back existing AFM_1 contamination. Therefore, the mean of all 192 samples (0.54 ng/g) may overestimate the effective average AFM_1 contamination levels. However, the

Table 3. Frequency distribution of aflatoxins in all analysed samples

AF total (ng/g)[a]	0–6.8[f]	–10	–15	–20	–25	–30	–35	–40	total samples	mean
rice, all	90	4	3	–	–	–	1	1	99	4.5
corn, soft[b]	42	4	1	–	–	–	–	–	47	4.0
beans	75	2	2	–	–	–	–	–	79	3.7

AF total (ng/g)[a,c]	0–6.8[f]	–20	–50	–100	–500	–2000	–4000	–6000	total samples	mean
peanuts	–	3	7	2	10	6	1	2	31	698
corn, hard[d]	26	6	1	2	4	3	–	–	42	110

AFM_1 (ng/g)[e]	0–0.125[f]	–0.50	–1.0	–1.5	–2.0	–3.0	–4.5	–6.0	total samples	mean
milk	49	80	30	18	8	5	1	1	192	0.54

[a] AF total by ELISA test kit «Veratox quantitative aflatoxin total test» from Neogen Corp., Lansing, MI
[b] Corn, soft refers to mountain region grown soft endosperm corn
[c] Where necessary, dilutions in the range of 1:10 to 1:100 were prepared by mixing sample extract of contaminated sample with sample extract of control sample, which tested negative in prior ELISA analyses.
[d] Corn, hard refers to coast grown hard endosperm corn as well as inter-Andean grown hard and medium hard endosperm corn.
[e] AFM_1 by ELISA test kit «Veratox quantitative aflatoxin M_1 test» from Neogen Corp., MI
[f] Detection limit (see Table 1)

mean value of 38 independently analysed samples was 0.44 ng/g and thus in the same range.

Factors influencing the occurrence of aflatoxin contaminations

During sampling, attention was paid to monitoring possible factors influencing the aflatoxin contamination levels. These factors include matrix, variety, climate region, moisture content, storage conditions, marketing, appearance, season (milk) and processing.

Variety and matrix

Huge differences between hard endosperm and soft endosperm corn were seen (table 3). Hard endosperm corn grown in the coastal, the eastern and inter-Andean tropical regions (24 samples, mean: 189 ng/g) proved to be a matrix with high probability of aflatoxin contamination. Thirty-eight percent of the samples analy-

sed showed aflatoxin contaminations above 50 ng/g and 13% even above 950 ng/g. In contrast, medium hard endosperm corn grown in the inter-Andean subtropical to cold region (18 samples, mean: 4.2 ng/g) was found to be only moderately contaminated, similar to soft corn grown in the mountain region grown (47 samples, mean: 4.0 ng/g).

For rice, no statistically significant difference was found between whole rice, polished rice, broken fraction, polish powder fraction and rice in husk. However, higher contaminations tended to occur in rice in husk and in polish powder fractions indicating that the major part of the aflatoxin contamination of rice can be separated into the polish powder fraction which is used for feeding purposes.

Among the bean varieties analysed, a statistically significant difference was found between soya beans (2 samples, $p \leq 0.0001$) and all other varieties. However, the two soya bean samples examined were of bad appearance (old), so that the difference could be based on other reasons than the variety.

In peanuts, no statistically significant difference could be detected between the varieties «Boliche» (7 samples, mean: 91 ng/g) and others (mainly «Charapoto», 21 samples, mean: 716 ng/g). Mixtures sold of both varieties (3 samples, mean: 1990 ng/g) had a markedly increased risk of high aflatoxin content. This might be due to the selling of old peanut stocks by merchants. The variety «Boliche» was introduced in 1991 (10). Since it is not known whether producers of this variety were instructed in more careful handling practices during harvest and storage, the observed tendency to lower AF contaminations might be due to other reasons than variety differences. Because in Ecuador harvesting (in-shell drying of peanuts in the field, often in bulk) and storage practices (shelled peanuts were seen in polypropylene bags during periods of marketing) are far from ideal, the problem of high aflatoxin contamination in peanuts clearly needs further investigation.

In Brazil, in-shell stored peanuts were found to be contaminated with aflatoxins between not detectable levels and 540 ng/g when stored in jute bags and between 354 ng/g and 5415 ng/g when stored in poypropylene bags. Furthermore, regional differences as well as differences from year to year were observed (18).

Climatic region

Polished rice sampled in the inter-Andean region (15 samples, mean: 4.7 ng/g) was not significantly different from polished rice sampled in the coast region (14 samples, mean: 3.7 ng/g). However, three out of 4 polished rice samples with aflatoxin contaminations above 7.4 ng/g originated from the installations of the ENAC in Cuenca and Quito (inter-Andean) and were harvested in the years 90/91 suggesting that other factors than climatic region might influence the contamination level, mainly possible field contamination of rice, duration of storage and the practice of drying down and cleaning-up rice, which got either infested by insects or was too humid during storage.

Hard corn grown in the tropical humid coast (11 samples, mean: 274 ng/g) and in the Orient (4 samples, mean: 352 ng/g) were more highly contaminated than in the inter-Andean region (18 samples, mean: 4.2 ng/g) with $p \leq 0.007$ (fig. 2)

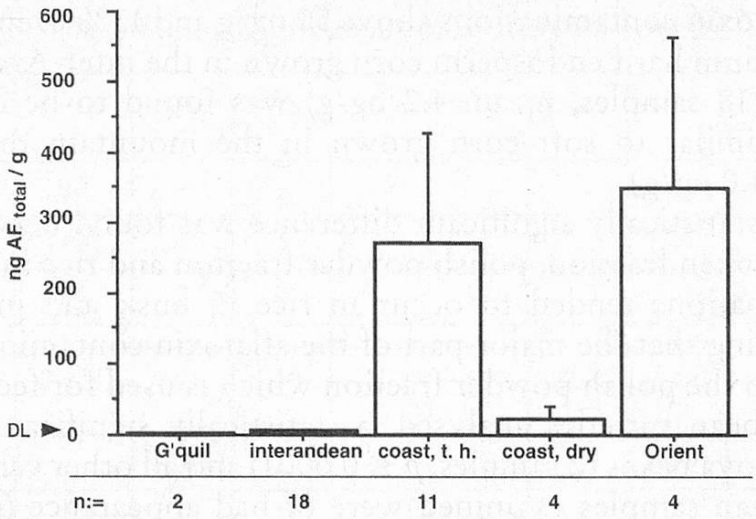

Fig. 2. Influence of climate region on aflatoxin contamination in hard corn

illustrating that the aflatoxin contamination was highest in the tropical humid regions of the coast and the east.

Interestingly, among the milk samples of regional collective consignments, 19 samples from inter-Andean subtropical to cold production regions (mean: 0.33 ng/ml) were statistically significantly different from the 10 samples of inter-Andean tropical and tropical humid coast production (mean: 0.12 ng/ml) with $p = 0.006$.

Moisture content

No commodity examined displayed statistically significant differences in this context, a fact that might be due to seasonal effects (equilibrium humidity of the sample with ambient relative humidity) or the above mentioned practice of drying down and cleaning-up commodities damaged (e.g. by humidity) during storage.

Storage conditions

No differences were found between the different storage conditions for rice, corn and beans, although the content of aflatoxins in hard endosperm corn samples taken in the field (4 samples, mean: 92 ng/g) seemed to increase until storage (2 samples, mean: 489 ng/g) and during unknown type of storage (6 samples, mean: 498 ng/g). Rice in husk coming from the field (11 samples, mean: 6.6 ng/g) lost about half of the aflatoxin content by polishing before storage (19 samples, mean: 3.9 ng/g). During storage in bag depots (30 samples, mean: 4.5 ng/g) as well as during storage in concrete silos (3 samples, mean: 13 ng/g), aflatoxin contamination seemed to increase. These tendencies might indicate that field contamination of rice with aflatoxin is overlayed with post harvest aflatoxin contamination during storage. Small farms usually dry down their rice to about 16% moisture content and store it in jute bags for approximately two months until winter rains finish and the climate allows adequate drying of rice. The influence of moisture content and storage conditions of rice on aflatoxin contamination levels was further examined.

First, field samples (rice in husk, 400 ears) from three different varieties were analysed and no aflatoxin contamination could be detected. Then, rice samples (60–800 kg from 5 varieties, each) were harvested. Subsequently, different harvesting conditions were simulated: a) drying rice down to 12% humidity immediately, b) simulating rainfalls by periodically wetting the crop over 10 days at maximal extent, c) drying down the rice to about 16% moisture content followed by storage under these conditions. Subsequently, rice was polished and different conditions of moisture content were prepared (12%, 13.5% and 15%) and stored at each of three different ambient conditions: 1) 30 °C, controlled conditions, 2) inter-Andean bag depot at ambient temperature, 3) coast bag depot at ambient temperature. Subsamples of 1–3 kg were taken and analysed by ELISA during 5 months. One sample from procedure c (rice in husk) contained 6 ng aflatoxins/g after two weeks but no contamination was found two months later. Polish powder fractions from procedure a (one sample) and b (2 samples) contained aflatoxins from 20 to 30 ng/g. None of the polished rice samples examined showed aflatoxin contents above 2 ng/g during the period of analysis. Thus, aflatoxin contamination seemed to affect mainly the pericarp and polished rice was rarely contaminated at high levels. Most likely, this contamination originates from the field but will only arise after heavy rainfalls (especially in years with the climate phenomenon «El Niño» present) or after prolonged storage (more than one year). However, *de novo* contamination during storage cannot be excluded.

Marketing

In hard and medium hard endosperm corn, samples obtained from market places (3 samples, mean: 917 ng/g) were statistically significantly different from all other types of marketing ($p \leq 0.003$): wholesale dealers and retail stores as «sale» (4 samples, mean: 108 ng/g), ENAC (8 samples, mean: 29 ng/g), farm (6 samples, mean: 183 ng/g) and FAO (18 samples of inter-Andean grown medium hard corn, mean: 4.2 ng/g). Thus, hard endosperm corn is often contaminated already at the farm level and little control exists during buying and selling activities from private people, in contrast to quite good control and selection criteria from the ENAC which, nevertheless, do allow an average contamination level of 20 ng/g to be reached. Interestingly, medium hard endosperm corn grown in the inter-Andean region did not tend to high levels of aflatoxin contamination, agreeing with a previous report (19).

No statistically significant difference was seen between milk originating from small and large scale farms. In and around Ingueza, large scale farms (17 samples, mean: 0.13 ng/ml) differed from small farms (37 samples, mean: 0.7 ng/ml) with $p = 0.0007$, but in and around Riobamba, the contrary situation was found: small farms (27 samples, mean 0.6 ng/ml) differed from large scale farms (10 samples, mean: 1.3 ng/ml) with $p = 0.014$.

Appearance

In hard and medium hard endosperm corn, mouldy corn (5 samples, mean: 623 ng/g) was statistically significantly different from corn judged nice (9 samples, mean: 6 ng/g), normal (11 samples, mean: 36 ng/g), insect infested (10 samples, mean: 5 ng/g) or bad (old, dirty) looking (4 samples, mean: 247 ng/g) with $p \leq 0.015$ (fig. 3). In addition, HPLC analysis revealed co-contamination with ochratoxin A at levels up to 250 ng/g (20). Thus, judgement of the appearance of hard endosperm corn in grains by the consumer can help to avoid aflatoxin contents above 100 ng/g, a level, however, which cannot guarantee harmlessness for animal or human health. Visibly mouldy soft corn (10 samples, mean: 3.4 ng/g) had less aflatoxin content than 4 samples judged to be of bad appearance (mean: 6.5 ng/g). Other analyses (20) revealed the occurrence mainly of deoxynivalenol (up to 1.4 µg/g) and ochratoxin A (up to 170 ng/g). Beans with bad appearance (11 samples, mean: 5.5 ng/g) were different from samples judged to be nice (21 samples, mean: 3.4 ng/g), dirty (4 samples, mean: 3.4 ng/g), mouldy (4 samples, mean 3.9 ng/g) and insect infested beans (7 samples, mean: 3.4 ng/g) with $p \leq 0.08$.

Decontamination of a peanut sample by hand sorting

The practice of sorting peanuts by hand is widespread in Ecuador, because peanuts represent a high price crop and are thus sold to the public containing dirt, waste and mouldy peanuts. Since peanuts may reach extremly high levels of aflatoxin contamination, the effectivity of hand-sorting was tested experimentally. From a 4.8 kg peanut sample containing 120 ng/g total aflatoxins from a local market place, 13% of waste (dirt, mouldy peanuts), 22% of damaged peanuts (grits, deformed, insect or slightly mould infested) and 13% of peanuts with coloured spots were removed sequentially. The corresponding aflatoxin contents of the remaining samples and those removed (in parenthesis) were 88 (460) ng/g after the first step, 34 (92) ng/g and 20 (70) ng/g after steps two and three, respectively. Thus, hand-sorting can effectively reduce the level of aflatoxin contamination of peanuts

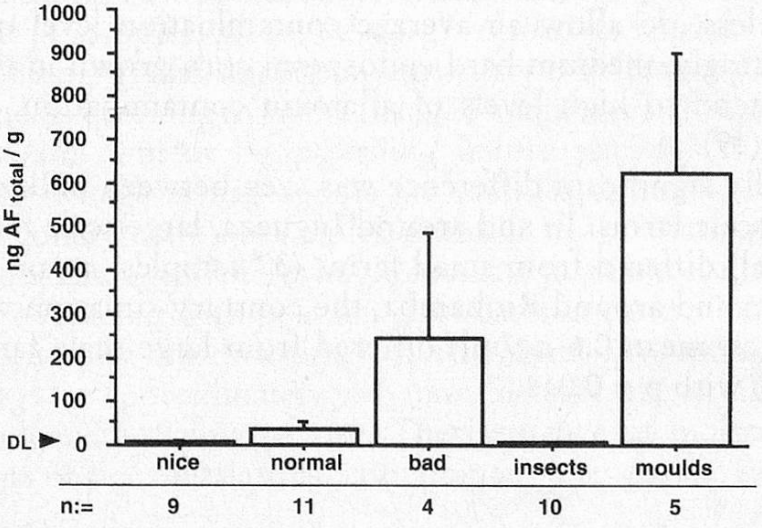

Fig. 3. Correlation of appearance with aflatoxin contamination in hard corn

several fold. Nevertheless, the remaining contamination level may still be relatively high and considering the high price character of the crop it seems doubtful that up to 50% of bought peanuts would be discarded. Obligate control and decontamination of peanuts by established procedures, e.g. with fluorescence detection of aflatoxins by UV-light, should be introduced (21, 22).

Influence of season on AFM_1 content in milk

No difference was seen in the AFM_1 content in milk between different seasons. This might be due to the limited number of samples from regional collective consignments or to steadily occurring changes at the producers level. Highest AFM_1 contents in milk were from March (6 samples, mean: 0.43 ng/ml), May (7 samples, mean: 0.29 ng/ml) and December (5 samples, mean: 0.28 ng/ml) and lowest levels were from January (3 samples, mean: 0.11 ng/ml) and October (7 samples, mean: 0.16 ng/ml).

Nixtamalisation of corn

Nixtamalisation (alkaline cooking) of corn samples is a widespread corn preparation technique in Central and South America, rooting back to Maya and Aztec civilisations (23). Nowadays nixtamalisation is conducted both in rural areas and at the industrial level (24) in order to produce «tortillas», «chips», «tamales» and other food for human consumption. Alkaline cooking is achieved by the use of either $Ca(OH)_2$ or ash (rural regions and poor people) at concentrations from 0.2 to 2% (w/w of corn).

Intermediate to hard endosperm corn should be used in order to obtain best quality products (25), but soft to hard corn can be used.

In Ecuador, an early maturing soft endosperm corn is used to produce «mote», which accompanies many typical dishes. The effect of nixtamalisation of corn on aflatoxin levels was tested experimentally as outlined in Materials and Methods. The overall reduction of aflatoxin levels of 60% obtained (table 4) is in accordance with literature (26), although no removal of aflatoxins (27) as well as an 80–100% removal (28) have been reported. Thus, alkaline cooking of corn only partially reduces the aflatoxin contamination levels. Acidification in the intestinal tract might partially restore aflatoxins (26). Regarding the decomposition of zearalenone and deoxynivalenol, contradictary reports exist (24). Definitively, more work is needed to decide about the real extent of destruction of different mycotoxins in different corn types after different alkaline cooking procedures.

Daily aflatoxin intake in Ecuador

For the calculation of the daily intake of aflatoxins by Ecuadorians, the mean and median values of aflatoxin contents of the foodstuffs were used (table 5). For the risk assessment, the mean values of consumption of the different food items are needed. From the foodstuffs examined, Ecuadorians eat approximately 110 g rice, 21 g soft corn, 10 g dried beans, 1 g peanuts and 280 ml milk (29). No statistics are available about the average daily consumption of hard endosperm corn. Informa-

Table 4. Nixtamalisation of corn samples

Origin of samples	AF total by ELISA in native corn (ng/g)	AF total by ELISA in alkaline-cooked corn (ng/g)	%-Reduction of AF total by ELISA with alkaline-cooking
Brazil 1[a]	11.7	1.6	86
Brazil 2[a]	12.7	2.2	83
Poultier 1[b]	7.4	4.0	46
Poultier 2[b]	4.1	3.5	15
Guayaquil	3.6	5.7[c]	(+58)
Orient A	975	430	66
Orient B	17.9[d]	12.9	28
Esmeraldas A	18.7	13.9	26
Esmeraldas B	1500	486	68
Total (mean):	283.5	106.6	62

[a+b] Samples were analysed in duplicate. Nixtamalisation procedure was unknown.
[c] Between analysis of native and alkaline cooked sub-samples, pretreated sample was thawed 4 times (overnight) for other analysis.
[d] Analysed by TLC

tion (when given) about the use of agricultural surface (9) show a 5 to 15% use for subsistence production, thus a percentage in that range could serve as an estimate of the consumption of hard endosperm corn, and an average daily intake of 10 g (7.5% of 490 000 tons of production) seems reasonable when taking into account

Table 5. Daily intake of total aflatoxin by Ecuadorian population (ng/kg b.w./d)

matrix	daily consumption g	arithmetic mean AF ng/g	median AF ng/g	daily intake based on mean AF ng/kg b.w./d	daily intake based on median AF ng/kg b.w./d
rice	110	4.5	3.4	9.0	6.8
corn, soft	21	4	3.4	1.5	1.3
corn, hard	10	110	3.4	20.0	0.6
beans	10	3.7	3.4	0.7	0.6
peanuts	1	698	236	12.7	4.3
milk[a]	280	0.4	0.2	2.2	1.0
			total:	46.1	14.6

[a] Thirty-eight independently analyzed milk samples were used for the risk assessment.

that about 3/4 of the production is used in animal (mainly poultry) feed (30). Tortilla prepared from precooked (without lime or ash, no nixtamalisation) hard endosperm corn meal is eaten all over the country and the production of a single important factory (Molinos Poultier S.A. in Latacunga) accounts for more than 3 g/day per capita. Especially poor, rural people from the coast and the east region eat «salprieta», a dish accompanying green banana, made of a mix (1:1) of peanuts and handmilled hard endosperm corn, both of subsistence production. Other products of human consumption, e.g. sweet potatoes, different nuts (coco) and spices (chili peppers), which might contain aflatoxins and form part of the Ecuadorian diet, were not part of this study, and no data about contamination levels in Ecuador were available from the literature. Wheat, which is mainly (about 90%) imported, was not analysed. Cassava tested negative in this as well as in an earlier study (Dr. Espin de Rivera, 1991, data from 1985, personal communication).

The average weight of Ecuadorians is about 55 kg. Based on the arithmetic means, the resulting values of aflatoxin (mainly AFB_1) intake by Ecuadorians (indicated in ng/kg body weight/day) through the different matrices were 9.0 (rice), 1.5 (soft corn), 0.7 (beans), 12.7 (peanuts), 20 (hard corn) and 2.2 (milk), totalling 46 ng total aflatoxins/kg bw/day (table 5). Calculations based on the median values led to a lower estimate of the daily intake in the order of 15 ng/kg bw/day. The annual incidence of liver cancer in the Ecuadorian population was 49 cases/100 000 habitants (31).

Risk assessment

For comparison, in Europe, the daily intake of aflatoxins is estimated to be 0.1 ng/kg bw/day (32) and falls into the range of the calculated virtually safe dose for humans of 0.04 to 0.14 ng AF total / kg bw/day (4). The incidence of primary liver cancer is approximately 1 case / 100 000 habitants (33), but differences between regions are reported. Thus, Europe is dealing with a hypothetical risk, which can only be calculated by extrapolation of data from toxicologically active doses. On the other hand, several epidemiologic studies, conducted in order to obtain information on the relationship of estimated dietary intake of aflatoxins to the incidence of primary human liver cancer, were realised in Uganda (34, 35), the Philipines (36), Swaziland (37), Kenya (38), Thailand (39, 40) and Mozambique (41). The evaluation of some of these data (42) showed that aflatoxin ingestion varied over a range from 3 to 222 ng/kg body weight per day, whereas annual liver cancer incidence varied from 2 to 35 cases / 100 000 habitants. There is a positive association between high intake of aflatoxins and high incidence of liver cancer, which can be expressed as a linear function of the log of dietary aflatoxin intake (43, 44). The estimated dietary aflatoxin intake of the Ecuadorian population (15 to 46 ng/kg bw/day) would lead according to the above mentioned function to a smaller liver cancer incidence than actually reported (31). Higher than expected incidence of liver cancer was reported from the Guangxi region of China (45). The relative liver cancer risk can be modulated by many factors. Among these factors, the occurrence of Hepatitis B virus (46) and consumption of alcohol (47) can increase the relative risk in combi-

nation with aflatoxins about 60 fold. In the Ecuadorian population, about 0.05% of the people have «viral Hepatitis» or Hepatitis B (48) and about 7.6% are alcoholics (49); consumption above 14 l of pure alcohol per year) and an additional 8.7% are heavy drinkers. Several factors can decrease the relative risk of the incidence of cancer, such as nutritional modulations like dietary protein deficiency (50).

Fisher rats exposed to doses of 50 ng/kg bw (51) have a statistically significant increased liver tumor incidence (+ 9%). Although similarity exists between the species rat and man in the development and presentation of liver cancer (52), the potency of aflatoxin B_1 in rat has been shown to overestimate greatly the human liver cancer rate in the United States (53). However, no safety factor between real daily intake of aflatoxins by Ecuadorians and a proven toxicologicaly active dose of the hepatocarcinogen exists. It clearly can be concluded that the intake of 15 to 46 ng aflatoxin/kg body weight per day poses a human health hazard and that aflatoxin ingestion is partially responsible for the highly prevalent liver cancer incidence in Ecuador.

Conclusions

The ingestion of aflatoxins in foods and feeds poses a problem for human and animal health in Ecuador. The content of aflatoxins in rice is of importance, because this is the most important staple food of the Ecuadorian population. In Venezuela, the introduction of a limitation to a maximum of 5 ng/g of total aflatoxins in rice meals was discussed (4). In Ecuador, it should be definitely determined whether the storage of rice for periods longer than one year might cause increased contamination levels of aflatoxins. Hard endosperm corn proved to be a matrix of high risk for high aflatoxin contamination levels, especially when grown and stored in tropical hot, humid regions of the country. Furthermore, co-contamination with ochratoxin A occurs (20), which is supposed to be a carcinogen itself (54) and to be involved in Balkan endemic nephropathy (55). Soft and medium hard endosperm corn as well as beans do not pose a first order problem concerning their aflatoxin contents due to the quantity of consumption as well as the level of contamination. Post-harvesting programme concerning these matrices should be guided to avoid the disastrous losses by insect infestation and rodents during storage (56, 57). Peanuts were found to be contaminated with very high aflatoxin levels, as determined by ELISA analysis. Although the method has been collaboratively studied for raw peanuts containing $\geq$ 30 ng AF/g (58), confirmatory analysis with TLC or HPLC analysis should be performed. If the present results are confirmed, inmediate action should be taken to protect the Ecuadorians from adverse health effects. The interesting hint, that the variety «Boliche» might be more resistant to aflatoxin contamination, should be carefully examined. Ecuadorian milk was found to be contaminated by AFM_1, but this data has to be further confirmed with TLC or

HPLC analysis. The AFM_1 levels analysed in milk are of particular interest, because they might pose a special risk to children.

The calculated aflatoxin ingestion by the Ecuadorians should be analysed in parallel by clinical investigations. It would be of particular interest to examine Ecuadorian high risk groups (such as poor farmers in the tropical coast region and the east with subsistence corn and peanut production or people with diagnosed liver cancer) for the presence of the established molecular dosimetry markers AFB-N^7-guanine (and AFM_1) in urine and AFB-albumin adducts in blood (59). This kind of analysis would lead to a better estimation of the health hazard caused by aflatoxins in Ecuador.

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Summary

In Ecuador, between 1992 and 1994, different foods (rice, corn, beans, peanuts and milk) were analysed for aflatoxin contamination by ELISA and TLC. Regression analysis of ELISA versus TLC measurements showed good agreement between both methods. Recoveries from artificially contaminated samples ranged from 71% (milk) to 112% (beans) in ELISA and from 77% (corn) to 82% (beans) in TLC. Whereas only moderate aflatoxin contamination levels were found in rice, soft corn and beans, very high levels were found in peanuts and hard endosperm corn. In peanuts, high aflatoxin contamination levels seemed to be variety dependent and influenced by bad post-harvest conditions. High aflatoxin concentrations were detected in hard corn produced in the regions of the tropical coast and the east (orient), and a correlation between moisture content and aflatoxin contamination level was found for this matrix. Aflatoxin M_1 (AFM_1) contamination of milk was found to be higher in the important inter-Andean production region than in tropical lowlands. Total daily aflatoxin intake of Ecuadorians was estimated to range between 15 and 46 ng/kg body weight. Typical Ecuadorian practices like hand-sorting of peanuts, hygienic quality judgement by appearance and moisture content analysis (corn and rice) as well as nixtamalisation (alkaline cooking) of corn lower the aflatoxin contamination, but cannot guarantee harmlessness of the foodstuffs. Therefore, basic regulatory and control actions should be taken.

Zusammenfassung

In den Jahren 1992 bis 1994 wurden total 490 Proben von Grundnahrungsmitteln (Reis, Mais, Bohnen, Erdnüsse, Milch) aus Ecuador auf den Totalgehalt von Aflatoxinen mittels ELISA und Dünnschichtchromatographie (TLC) untersucht. Eine Regressionsanalyse ELISA versus TLC-Analysenwerte ergab eine befriedigende Übereinstimmung der beiden Methoden. Die Wiederfindungsrate von zugesetzten Aflatoxinen lag beim ELISA zwischen 71% (Milch) und 112% (Bohnen) bzw. 77% (Mais) bis 82% (Bohnen) bei TLC. Die höchsten Aflatoxingehalte wurden in Erdnüssen (bis 6 ppm; mg/kg) und Hartmais (bis 3 ppm) nachgewiesen. Die Reisproben enthielten vereinzelt bis zu 40 ppb (µg/kg), Weichmais und Bohnen bis 15 µg/kg an Gesamtaflatoxinen. Die Aflatoxinkontamination bei Erdnüssen erwies sich als sortenabhängig und abhängig von den Nacherntebedingungen. Die Maisproben mit den höchsten Aflatoxingehalten stammten aus der (tropischen) Küstenregion und der Ostregion Ecuadors, wobei eine positive Korrelation zwischen Feuchtigkeitsgehalt der Proben und Höhe der Aflatoxingehalte festgestellt werden konnte. Total 143 von 192 Milchproben enthielten Aflatoxin M_1 mit Gehalten bis zu 6 ppb (µg/l; Mittel 0,54 µg/l). Die mittlere Aflatoxinaufnahme wurde aufgrund dieser Daten und der Ernährungsgewohnheiten in Ecuador abgeschätzt: sie liegt im Bereich 15–46 ng/kg Körpergewicht und Tag und damit mehr als 100mal höher als in europäischen Ländern. Massnahmen zur Verbesserung der Situation sind dringend notwendig.

Résumé

Entre 1992 et 1994, la teneur totale en aflatoxines de 490 échantillons d'aliments de base (riz, maïs, haricots, cacahuètes et lait) provenant de l'Equateur a été déterminée par ELISA et par chromatographie sur couche mince (TLC). L'analyse statistique comparative utilisant la régression, démontre une concordance des résultats obtenus par ELISA et TLC. Les taux de récupération trouvés dans des échantillons contaminés intentionnellement se situent entre 71% (lait) et 112% (haricots) avec la technique ELISA, et entre 77% (maïs) et 82% (haricots) avec la méthode de TLC. Tandis que le degré de contamination par aflatoxines mis en évidence dans le riz (40 ppb; µg/kg), le maïs doux et les haricots est faible (15 µg/kg), la concentration de ces substances est très élevée dans les cacahuètes (6 ppm; mg/kg) et les grains durs de maïs (3 ppm). Il semblerait que la contamination importante des cacahuètes dépende de la variété et des conditions de stockage. Des teneurs en aflatoxines élevées ont été détectées dans les grains durs de maïs cultivés dans les régions de la côte tropicale et à l'est (orient), une corrélation existe entre l'état hygroscopique et le degré de contamination par aflatoxines de ces denrées alimentaires. La contamination du lait par aflatoxine M_1 (AFM_1) est plus élevée dans la région principale de production des hauts plateaux intérieurs et des bassins intramontagnards des Andes que dans les plaines tropicales. 192 échantillons de lait ont été analysés, 143 échantillons contiennent jusqu'à 6 ppb (µg/l; moyenne 0,54 µg/l) d'aflatoxine M_1. L'absorption journalière totale d'aflatoxines par les equatoriennes et equatoriens atteint des valeurs moyennes estimées entre 15 et 46 ng/kg de poids du corps, cette absorption est donc 100 fois plus élevée que dans les pays européens. Des pratiques typiques equatoriennes comme le triage à la main des cacahuètes, l'appréciation de la qualité hygiénique sur la base de l'apparence et la détermination de la teneur en humidité (maïs et riz), ainsi que la cuisson en milieu alcalin («nixtamalisation») de maïs, réduisent le degré de contamination par

aflatoxines, mais ne peuvent pas garantir l'innocuité de ces aliments. Par conséquent, des mesures au niveau légal et dans le domaine assurance qualité devraient être prises.

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