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Ochratoxin A levels in plasma from healthy blood donors from Valencia and estimation of exposure degree: comparison with previous national Spanish data

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Abstract

Blood plasma levels of ochratoxin A, a toxic secondary metabolite of several fungal species belonging to the genera *Aspergillus* and *Penicillium*, were determined in 168 blood donors from Valencian Community (Spain), using LC-FLD. In conjunction with the blood collection, detailed information about the diet was obtained by using a questionnaire that included a wide range of products potentially contaminated with the toxin. The investigation revealed a detection frequency of 100%. Mean level was 1.09 µg OTA/l of plasma and concentrations ranged between 0.15 and 5.71 µg OTA/l of plasma. Men's levels were slightly higher than levels observed in women. Results were analysed by Spearman rank correlation test and Gamma correlation statistic. There was no strong correlation between individual consumption of 26 food groups, described as possibly contaminated with OTA and plasma level of OTA. Multiple regression models and factorial regression models were obtained explaining 14.9% and 64.4% respectively, but they could not explain the overall of the variability. The dietary daily intake of ochratoxin A was calculated on the basis of plasma toxin levels using Breitholtz and Klassen formulas. For the overall population, mean values were 1.47 ± 1.25 and 2.16 ± 1.88 ng/kg b.w/day respectively. All results have been compared with other Spanish data.

Introduction

Ochratoxin A (OTA) is a toxic secondary metabolite of several fungal species belonging to the genera *Aspergillus* and *Penicillium* (van der Merwe et al., 1965; Pitt, 1987; Medina et al., 2005a).

OTA contaminates agricultural products and due to its long half life it accumulates in food, representing a serious threat to human and animal health throughout the world since it has been implicated in a diverse range of toxicological effects (O'Brien and Dietrich, 2005). OTA produces an inhibition of protein synthesis and lipid peroxidation by oxidative processes (Gautier et al., 2001; Omar et al., 1996). These mechanisms may generate nephrotoxic, neurotoxic and immunotoxic effects (Luhe et al., 2003; Schaaf et al., 2002; Álvarez et al., 2004). Teratogenic and carcinogenic effects have been described in some animal species (Veselá et al., 1983; Lioi et al., 2004). The IARC has classified OTA as a “possible human carcinogen (Group 2B)” based on sufficient evidence for carcinogenicity in experimental animal studies and inadequate evidence in humans (IARC, 1993). The toxic-kinetic parameters of OTA have not been well established in humans, but OTA showed high availability after oral administration in some animal studies (Fuchs and Hult, 1992; Omar et al., 1996).

OTA binds rapidly and with high affinity to plasma proteins with increased $T_{1/2}$ values (Mally et al., 2005). The metabolism in humans is basically produced through cytochrome P-450 (Dortant et al., 2001) generating less toxic metabolites (Fazecas et al., 2005) which are eliminated in urine and faeces (Li et al., 2000, 1997). OTA can cross the placenta and is also excreted in animal milk (Valenta and Goll, 1996; Breitholtz-Emanuelsson et al., 1993).

As a consequence of the possible health hazards related to the ingestion of OTA, efforts to assess OTA levels in foods have been made by researchers worldwide and by governmental authorities in many countries. Several authors have described contamination of OTA in a wide range of products as cereal, beans, roasted coffee and cocoa, malt and beer, bread and bakery products, wines and grape juices, spices, poultry meat and kidney, pig kidneys and pork sausages (Van Egmond and Speijers, 1994; Jorgensen, 1998; Vrabcheva et al., 1998, Otteneder and Majerus 2000, 2001; Castellari et al., 2000; Thirumala-Devi et al., 2001; Thuvander et al., 2001; Visconti et

al., 2000; Leitner et al., 2002; Medina et al., 2005b, 2006; Mateo et al., 2007). It has also been found in animal tissues, muscle, and organs, eggs (Marquardt and Frolich, 1992; Valenta, H., 1998; Moreno Guillamont et al., 2005), cow's and human milk (Zimmerli and Dick, 1995; Valenta, H., 1998; Breitholtz-Emanuelson et al., 1993).

There are numerous reports from many countries in the world describing high frequencies of human blood contamination by ochratoxin A, although at low levels, showing widespread human exposure to this mycotoxin in Europe (Petkova-Bocharova et al., 1988; Breitholtz et al., 1991; Goliński et al., 1991; Hald, 1991; Petkova-Bocharova and Castegnaro, 1991; Creppy et al., 1993; Hadlok, 1993; Ruprich and Ostrý, 1993; Breitholtz-Emanuelson et al., 1994; Kovács et al., 1995; Zimmerli and Dick, 1995; Solti et al., 1997; Domijan et al., 1999; Palli et al., 1999; Peraica et al., 1999; Ozcelik et al., 2001, Thuvander et al., 2001), in North America (Kuiper-Goodman et al., 1993; Scott et al., 1998), in Japan (Ueno et al., 1998), in Africa (Kane et al., 1991; Singare-Tigori et al., 2006) and in Asia (Assaf et al., 2004). Roughly, ochratoxin A levels were found to be at the $\mu\text{g/l}$ range, often with frequencies among population extending from 6.6% to 100% (Marquardt and Frolich, 1992; Thuvander et al., 2001; Zimmerli and Dick, 1995) which also depends on the level of the limit of detection of the method carried out for analysis in sera or plasma samples.

The evaluation of OTA levels in different types of populations is unquestionable to provide data on exposure and evaluate the possible human health risk to OTA.

Over the last 12 years four studies have been published analysing levels of OTA in human blood. Burdaspal and Legarda and Jimenez et al. in 1998, Perez de Obanos et al. in 2001 and recently Coronel et al. in 2009.

Moreover the scarce of works done in Spain, generally, most of the studies published regarding other countries just show the OTA incidence in human blood serum or plasma but the relationship between the plasma levels of OTA, the estimated dietary intake and the possible correlation with the consumption of different food and beverages has been rarely studied.

In the present paper, we developed i) an evaluation of the consumption of foods and beverages considered as potentially contaminated with OTA in a wide number of healthy blood donors from the Valencian Community population (Mediterranean region) in Spain, ii) quantification of OTA plasma levels from these

healthy blood donors iii) a study of the potential correlation between the OTA levels in plasma and the diet, iv) the calculated dietary intake and v) a comparison with data obtained in previous studies made in different Spanish areas.

Materials and methods

Sampling and description of populations

Blood plasma samples were collected from healthy blood donors at the “Centro de Transfusiones de la Comunidad Valenciana” (CTCV) Valencian Community Transfusion Centre, Valencia, Spain, from 1st July to 14th of November 2008. The blood samples were taken in connection with the ordinary collection of blood, and all blood donors were informed and invited to participate in the survey after giving their informed consent. Ethylenediaminetetrachloroacetic acid (EDTA) was used as an anticoagulant. After centrifugation, the 3ml plasma samples were immediately frozen at -70°C. Different tests were made with the original plasma as routine in the CTCV to ensure absence of infectious agents. Once the results were obtained, samples which were found to be free of any infectious agents were then forwarded on dry ice to the Valencia University Fungi and Mycotoxin Research Laboratory, for OTA analysis.

Collection of individual food consumption data

In conjunction with the blood collection, detailed information about the diet of the healthy blood donors was obtained. The questionnaire included information about the consumption of a wide range of products such as meat (chicken, turkey, beef, pork, ...), beverages (beer, wine, fruit juices, coffee), cereal derived products (bakery products, pasta,...), spices, cheese, chocolate, etc. Additional information about the typical amount eaten of each item, consumption frequency or origin (supermarket, retails shops...) of all food items was collected. Furthermore some question regarding the age, sex, job, consumption of diet and bio-products, meals in restaurants... were done to all the healthy blood donor subjects that agreed to participate in the study.

Standards and reagents

The ochratoxin A standard was purchased from Sigma (Sigma-Aldrich, Alcobendas, Spain). A stock solution of about 500 mg/l was prepared by solving 1mg of OTA in 2ml

of toluene–acetic acid (99:1, v/v). A series of working standards was prepared by evaporation of known aliquots of the stock solution and dissolution in filtered LC mobile phase. They were used to calibrate the detector response.

The concentration of the stock solution was determined by measuring absorbance at 333 nm of a diluted solution (20–30 mg/l) of OTA in toluene-acetic acid (99:1, v/v). Acetonitrile, chloroform, and methanol (all LC grade), acetic acid (A.R.) and phosphoric acid (85%, A.R.) were from J.T. Baker (Deventer, the Netherlands). Sodium chloride and dimethyl sulfoxide were purchased from Sigma (Sigma-Aldrich, Alcobendas, Spain). Pure water was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA) and was used when water was required.

Extraction, clean-up and analysis of ochratoxin A

Extraction was made following the protocol used by Lino et al. (2008) with some modifications. Briefly, two mL of plasma were added of 1 mL of a solution containing 33.7 mL of 85% orthophosphoric acid and 118 g sodium chloride per liter, and mixed for 1 min. After, 2 x 4 mL of chloroform were added and mixed for 20 min using a rotary mixer. The organic extracts were reunified and concentrated to dryness at 45°C under gentle stream of nitrogen. The residue was dissolved in 0.25 mL of water: dimethyl sulfoxide (70:30, v/v) and transferred to an amber glass vial. One hundred µl were injected into the LC system.

The LC system consisted of a Waters 600E system controller, a Millipore Waters 717 Plus autosampler and a Waters 470 scanning fluorescence detector (Waters, Milford, Ma, USA). Excitation and emission wavelengths were 330 and 460 nm, respectively. The samples were separated using a C₁₈ Zorbax Eclipse Plus[®] (150 x 4.6 mm, 3.5 µm) (Agilent Technologies, Waldbronn, Germany), with a guard column of the same material. Run time for samples was 20 min with OTA being detected at about 12 min. The flow rate of the mobile phase (water-acetonitrile-acetic acid; 59:39:2, v/v/v) was 1 mL min⁻¹.

Analysis of the results was carried out on a computer with Millennium[®] 4.0 software (Waters).

Calibration lines were constructed using blood plasma spiked with different amounts of OTA standard in the range 0.05–10 µg OTA/l of medium. Since OTA free samples

were not found, calibration lines were constructed with the standard addition method using a 50ml sample supplied by CTCV. The interval trend line obtained fitted perfectly with an $R^2=0.997$. For each point three replicates were made.

Method performance

The limit of detection (LOD) was calculated using the IUPAC formula (Long and Winefordner, 1983):

$$LOD = \bar{X}_n + 3 \cdot S_{n-1}$$

Where, \bar{X} is the concentration mean and S_n the standard deviation. The limit of quantification (LOQ) was calculated according the next equation:

$$LOQ = \bar{X}_n + 10 \cdot S_{n-1}$$

The results have shown a LOD of 0.01 µg OTA/l blood plasma, and a LOQ 0.07 µg OTA/l. All these determinations were carried out in triplicate.

The OTA recovery rate from blood plasma was calculated (using another 50 ml sample as in the previous section) by addition of known amounts of OTA in the range of 0.1 to 5 µg OTA/l obtaining a mean recovery of 81.6±10.9 %. All the results presented have been corrected according to this value.

Repeatability was tested in the same day on five replicates at the spiking level of 0.5 µg OTA/l, and presented a relative standard deviation of 11%. Reproducibility was assessed in three different days also at 0.5 µg OTA/l, and the relative standard deviation was 10%.

LC-MS/MS confirmation of OTA in plasma samples

Once the LC-FLD data were collected, medium-high concentration samples were selected and the extract was dried in low volume chromatograph vials, using a gentle stream of N₂ at room temperature. Once they were near to be dry, 75 µL of methanol: water (1:1 v/v) were added and 50 µL were injected in the LC-ESI-MS/MS system.

LC-triple quadrupole mass spectrometry (LC-MS/MS) was used to confirm the presence of OTA in plasma samples. Transition between 403.8 *m/z* ion and 358.2 *m/z* ion was investigated. OTA was detected in all concentrated samples containing more than 15

ng OTA/ml (LOD) or above, confirming the results obtained previously by LC-FLD for samples containing more than 0.40-0.45 µg OTA/l of blood.

The analysis was carried out on a Shimadzu LC-10AD (Shimadzu Corporation, Kyoto, Japan), and an automatic injector Shimadzu SIL-10AS equipped with a C₁₈ Teknokroma Mediterranea Sea18 (50 mm x 2.1 mm, 5 µm) (Teknokroma, Sant Cugat del Valles, Spain) at room temperature and coupled to a Micromass Quattro™ triple-quadrupole mass spectrometer (Waters, Beverly, MA, USA). The mobile phase was programmed following a linear gradient at a flow rate of 0.3 mL/min. Solvent A was water with 0.5% formic acid, and solvent B was methanol with 0.5% formic acid. The gradient program was as follows: 0-5 min, 60% A; 2 min, 10% A; 8 min, 2 % A; 0.5 min, 60% A. Injection volume was 20 µL.

Micromass Quattro™ triple-quadrupole mass spectrometer was equipped with a Z-spray electrospray ionization source. Electrospray ionization method was performed using the following ionization source parameters: Capillary: 3.5 KV, skimmer: 2 V, RF Lens: 0.5 V, source temperature: 120 °C, desolvation temperature: 150 °C. MS1 parameters were LM resolution, 13; HM resolution, 13; ion energy, 1. MS2 parameters were LM resolution, 13; HM resolution, 13; ion energy, 1; multiplier 650 V.

Spectra were obtained in positive ionization multiple reaction monitoring (MRM) mode.

Confirmation by ESI-MS/MS was based on the transition between parent ion $[M + H]^+$ and the daughter ion $[(M + H)^+ - \text{HCOOH}]$, whose m/z ratios are 403.8 and 358.2, respectively.

Calculation of ochratoxin A intake based on plasma levels

The dietary daily intake of ochratoxin A was calculated on the basis of plasma toxin levels using the equation $k_0 = Cl_p \cdot C_p / A$, where k_0 is the dietary intake (ng/kg body weight and day) (Breitholtz et al. 1991). Cl_p is the plasma clearance (mL/kg body weight and day), C_p is the plasma concentration of ochratoxin A (ng/mL), and A is the bioavailability of the toxin. Cl_p and A were estimated at 0.67 mL/kg body weight/day and 0.5 (i.e. 50%), respectively, by Breitholtz et al. (1991), and these figures were used in the present calculations.

Moreover, the dietary daily intake was calculated according to the Klassen equation (Directorate General-Health and consumer protection, 2002; Thuvander et al., 2004). In these later studies Cl_p was estimated in 0.485 mL/kg body weight/day; a value lesser than the obtained by Breitholtz et al. (1991).

Statistical analysis

Statistical analysis was performed using the package STATISTICA 8 (StatSoft® Inc., 2007. Tulsa, OK, USA).

Data on ochratoxin A in plasma/BMI were tested for normality using standardized skewness and standardized Kurtosis tests. In both cases values were not within the range expected for data from a normal distribution. The correlation between plasma levels of the toxin and food consumption was investigated, due to non-normality, using the Spearman rank correlation test and *Gamma* statistic. Spearman R is useful assessing whether the two quantities we measure actually are varying together as predicted by our hypothesis, or whether any apparent correlation produced is just due to the amount of variation in results we could expect from chance circumstance. In short, *Gamma* is a probability; specifically, it is computed as the difference between the probability that the rank ordering of the two variables agree minus the probability that they disagree, divided by 1 minus the probability of ties.

STATISTICA 8 General Regression Module (GRM) was also used to build mathematical models to extract additional information from the data. This module allowed us to build models for designs with single-degree-of-freedom effects for continuous predictor variables using Sigma restricted parameterization. GRM implements stepwise and best-subset model-building techniques and uses the least squares methods of the general linear model to build models and to estimate and test hypotheses about effects included in the final model.

Multiple regression models, that do not study the effects between the different predictors, and factorial models, analyzing the above effects, were built.

Results

Dietary intake questionnaire

Meat product consumption, cereal and derived cereal consumption; beverages and other food items are shown in tables 1 to 4, respectively.

The main differences between men and woman food consumption were found on the breakfast cereals, beer and wine.

Women, among all age ranges, use to eat more breakfast cereals than men. In the case of the alcoholic beverages (wine and beer) the frequency of consumption is higher in men but also the average quantity is approximately 2-fold bigger in men group.

Ochratoxin A levels in plasma samples

A total of 168 samples of plasma were analyzed under the described conditions and OTA was detected in all of them at concentrations ranging between 0.15 and 5.71 μg OTA/l of plasma. Mean level was 1.09 μg OTA/l of plasma. Table 5 shows the Body Mass Index (B.M.I.), OTA level (μg /l plasma), Standard Deviation (S.D.) and the OTA ranges in the blood samples from the populations of men and women studied. In order to simplify the interpretation, data have been split in 3 different groups according to the age (18-35, 36-45 and over 45 years old). As it is shown in Table 5, level of OTA in men's blood plasma was slightly higher than levels observed in woman's blood plasma. Due to the non-normality of OTA level, data normalization was made by calculating the logarithm and then ANOVA was performed in the transformed variable using sex as categorical factor. No significant statistical differences were found in log(OTA blood levels) ($P=0.216$) between men and women populations.

Evaluation of daily intake

The dietary daily intake of ochratoxin A was calculated on the basis of plasma toxin levels using Breitholtz and Klassen formulas. For the overall population, mean values were 1.47 ± 1.25 and 2.16 ± 1.88 ng/kg b.w/day respectively.

Splitting the cases by sex and using both formula, Breitholtz and Klassen, the results were 1.52 ± 1.29 and 2.24 ± 1.90 ng/kg b.w/day for men and 1.31 ± 1.23 and 1.93 ± 1.81 ng/kg b.w/ day for women.

Performing calculations with the Breitholtz's formula, 3.0% of cases showed values higher than 5 ng/kg b.w/day. With Klassen formula, 6.7% of cases showed higher values.

Correlation between consumption of individual foods and plasma levels of ochratoxin A.

Correlations between individual consumption of each 26 food groups described as possibly contaminated with OTA and plasma level of OTA/BMI, and some data about habits, were examined. This transformation was done in order to relate the amount of OTA in plasma with the possible total volume of blood that is between 2800 and 3800 ml/m² body surface.

Results for OTA/BMI variable analyzed by Spearman rank correlation test and *Gamma* correlation statistic are shown in Table 6.

In the overall population, weak correlations were seen between plasma levels of OTA and beef, turkey, cold meat and bread consumptions. Results were the same in both tests performed.

Also a weak correlation was found between the plasma levels of OTA and people that use to have meals outside home.

Regarding men population, Spearman rank correlation test showed weak correlations with turkey, cold meat and bread consumptions. In this case, beef consumption wasn't statistically significant and neither outside home meals. Results of *Gamma* correlation statistic, that is preferable to Spearman *R* when the data contain many tied observations, were slightly different in this case and showed the same results obtained for the overall population.

Study of women population showed different results than the obtained for men. In these case Spearman rank correlation test and *Gamma* correlation statistic showed weak correlations between plasma levels of OTA and wine and cheese consumption.

Multiple regression models were obtained to extract more information about the data. As first step multiple regression methods were created using *Backward stepwise* model building (P to enter: .1; P to remove: .2). Using that methodology, main factors affecting the variability of OTA/BMI were found to be: wine, beer, bread and turkey factors, and also bun, fruit juice and Italian pasta consumption, despite they had less influence.

With this first approximation and building the whole model, the adjusted R² (determination factor) was 0.1493. Therefore, only 14.9% of the total variation in OTA

level/BMI can be explained by the linear relationship between the factors described above.

Next step was the building of factorial regression models. In this case, interaction between factors was considered. Five different set of 20% of the raw data were obtained randomly and were used for cross-validation. When models were built these 5 new variables were used to perform the model validation.

Models built included the main affecting factors obtained in multiple regressions. Other factors were also included and tested using *Backward stepwise* model building changing enter and remove condition in order trying to improve the model fitting.

Finally a third order, cross validated, whole model was built with factors: overall meat consumption, bread, bun, Italian pasta, beer, wine, coffee, fruit juices and dried fruit consumptions.

The adjusted R^2 obtained in this case was 0.6438, meaning that from the overall variation in plasma OTA level/BMI, only 64.4% of variability could be explained using the model built with the above factors. The other 35.6% of the total variation in OTA level/BMI remained unexplained.

Discussion

In this work, original results regarding consumption of foods considered as ochratoxin A vectors in the Valencian Community, wide area in the east of Spain where the Mediterranean diet is common, are described. Moreover, the OTA levels of a large sample of healthy individuals from this population are shown.

Incidence of OTA in plasma has been reported in subjects from different geographical parts of Spain, but the dietary habits and climate in many regions are very different.

The mean level we found in the Valencian Community (1.09 μg OTA/l) is lower than levels found in Madrid (1.19 ng/mL) (Burdaspal and Legarda, 1998) and higher than levels found in Lleida (0.86 μg OTA/l) (Coronel et al., 2009), in Navarra (0.71 μg OTA/l) (Jimenez et al., 1998) and Granada (0.63 ng/mL) (Pérez de Obanos et al., 2001).

These differences could be well explained attending to diet differences between these areas. However it is impossible to compare and discuss it because diet parameters of the analysed subjects were not studied in the other studies carried out in Spain or data used have been extracted from literature (Coronel et al., 2009).

Studies carried out in other countries as Portugal, Chile and Croatia also have shown differences in the OTA levels of populations belonging to different areas in the same country (Peraica et al., 2001; Muñoz et al., 2006; Lino et al., 2008).

Comparing our results with others from different countries, the obtained levels in plasma samples of healthy blood donors from the Valencian Community are similar to samples from Canada, United Kingdom (0.88 and 1.09 respectively), lower than values reported in Tunisia and Denmark (Hald, 1991; Hassen et al., 2004) and higher than levels reported in other countries as Italy, Morocco, Lebanon, Croatia, Sweden, Poland and Portugal (Assaf et al., 2004; Brera et al., 1999; Fillali et al., 2002; Hald, 1991; Lino et al., 2008; Peraica et al., 1999, 2001; Thuvander et al., 2001).

Also we found a higher frequency of positive samples (100%) in comparison with other studies carried out in Spain. Jimenez et al. (1998) found 53% of positives. Pérez de Obanos et al. (2001) found 72% and recently Coronel et al., (2009) have found 98.6% of positive samples. Our results agree with percentage found by Burdaspal and Legarda (1998) that found 100% of positives. These differences could be explained attending to the different analytical method limits of detection and quantification in the studies. These authors reported a limit of detection of 0.075 µg OTA/l (Coronel et al., 2009), 0.21 µg OTA/l (Pérez de Obanos et al., 2001) and 0.52 µg OTA/l (Jimenez et al., 1998), whereas the limit of detection of Burdaspal and Legarda (1998) was 0.020 µg OTA/l and in this study was 0.010 µg OTA/l of blood plasma.

In the literature, frequencies between 6.6% and 100% of samples with presence of OTA have been reported and explanations for these differences have been found in the different LOD and LOQ as well (Lino et al., 2008; Jimenez et al., 1998; Thuvander et al., 2001).

Lack of differences between OTA plasma levels in men and women agrees with results found in Lleida by Coronel et al. (2009), and with other studies in other countries (Breitholtz et al., 1991; Thuvander et al., 2001; Ueno et al., 1998). Even though other authors described significant differences in OTA blood plasma levels regarding sex (Muñoz et al., 2006).

We did not find differences between age groups and these results do not agree with those obtained by Coronel et al. (2009) in Spain and in other countries as Portugal (Lino et al., 2008). Our results agree with the study carried out by Jimenez et al. (1998)

in Navarra (Spain) and other studies carried out overseas (Ueno et al., 1998; Palli et al., 1999; Gareis et al., 2000; Grosso et al., 2003; Skaug, 2003; Assaf et al., 2004) that showed no differences among age groups. Although these results should be interpreted with care due to in each case the age groups were defined differently.

The dietary daily intake was calculated from plasma levels using two different formulas. Basically the calculation is the same but the figure of the plasma clearance (Cl_p) is lesser using the Klassen approach. This leads to higher calculated ochratoxin daily intakes. Despite the fact the means obtained with both formulas (1.47 ± 1.25 and 2.16 ± 1.88 ng/kg b.w/ day, respectively) were below the tolerable daily intake, 5 ng/kg b.w, estimated by the Scientific Committee on Foods (SCF) of the European Union in 2002, individual values higher than the limits have been found. In the case of Breitholtz's formula, 3.0% of cases showed higher values and with Klassen formula, 6.7%. That high levels found may cause concern, once OTA is classified as possible carcinogen for human (group 2B) by International Agency for Research on Cancer (IARC, 1993).

Although the study involved a high number of subjects and a high number of food items that often are contaminated with ochratoxin A, the relation between the OTA plasma levels and these food items were weak.

Correlations tests performed did not show stronger correlation between the OTA level in plasma and some variables, despite of some of them resulted statistically significant, the correlation coefficients or probabilities were very small. Clear differences were observed between the men and women groups. These differences show that maybe each individual or group of individuals has different main sources of OTA.

Investigation on possible effects between variables was done building regression models and in this case some variables and their combinations explained around the 60% of the variability among the data.

Models could not explain the overall of the variability and when some samples were removed from the model in order to perform cross validation samples some of them were not well predicted by the model, despite of most of them were well predicted. These results mean that some variability sources are being ignored in the model. Also it should be taken into account the different sources of uncertainty that this kind of studies has. Apart from the analytical uncertainty typical in all mycotoxin analysis it is

possible that there are some mistakes in data from the questionnaires, possible bias during the sampling as only volunteer people was included and because there are seasonal differences regarding the food consumption; we did not consider other sources of ochratoxin A, for example via airborne particles and spores from ochratoxigenic fungi (Brera et al., 2002).

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Table 1- Meat and meat-derived products frequency of consumption, from healthy blood donors living in the Valencian Community, in times per week. Data collected from 168 questionnaires are shown in overall and also split by sex and age.

	Age range	Number of cases	Overall meat consumption (times/week)	Pork consumption (times/week)	Beef consumption (times/week)	Chicken consumption (times/week)	Turkey consumption (times/week)	Other meats consumption (times/week)	Animal entrails (% of Yes) [average frequency among positives]	Cold meat consumption (times/week)	Sausages consumption (times/week)
All data		168	4.88	1.55	1.26	2.02	0.54	0.48	27.9 [2 or 3 days/month]	1.98	1.27
Men											
	Overall	120	4.93	1.66	1.34	1.93	0.43	0.53	28.3 [2 or 3 days/month]	2.19	1.54
	18-35	42	5.85	2.07	1.38	2.19	0.62	0.67	33.3 [2 or 3 days/month]	2.55	2.09
	36-45	53	4.77	1.62	1.36	1.77	0.42	0.38	20.8 [3 or + days/month]	2.11	1.36
	46-	25	3.72	0.95	1.21	1.71	0.17	0.67	36.0 [1 day/month]	1.79	0.92
Women											
	Overall	48	4.77	1.27	1.06	2.27	0.79	0.33	27.1 [2 or 3 days/month]	1.46	0.60
	18-35	24	5.00	1.54	1.04	2.08	1.17	0.25	29.2 [3 or + days/month]	2.04	0.58
	36-45	13	4.30	1.00	1.08	2.53	0.39	0.15	23.1 [2 or 3 days/month]	1.46	0.69
	46-	11	4.81	1.00	1.09	2.36	0.46	0.73	27.3 [1 day/month]	0.18	0.55

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Table 2- Cereals and derivates frequency of consumption, from healthy blood donors living in the Valencian Community, in times per week and average quantity of amount consumed each time. Data collected from 168 questionnaires are shown in overall and also split by sex and age.

	Age range	Number of cases	Days of bread consumption / week [Avg. quantity in grams]	Days of bun consumption / week [Avg. quantity in grams]	Days of Italian pasta consumption / week [Avg. quantity in grams]	Days of breakfast cereal consumption/ week [Avg. quantity in grams]	Days of cookie consumption/ week [Avg. number of cookies]	Days of sweet corn consumption/ week [Avg. quantity in grams]	Days of snacks and crisp consumption/ week	% people that selected whole-grain flour [% people that selected both plain and wholegrain]
All data		168	6.06 [129.75]	1.27 [32.6]	2.02 [99.82]	1.36 [11.6]	2.19 [2.2]	1.28	0.90	14.3 [4.2]
Men										
	Overall	120	6.09 [150.5]	1.39 [35.8]	2.08 [103.1]	0.98 [10.0]	2.23 [2.9]	1.26	0.87	14.2 [4.2]
	18-35	42	5.98 [147.9]	1.71 [46.7]	2.09 [105.4]	1.48 [13.6]	2.21 [3.1]	1.43	1.33	4.16 [1.7]
	36-45	53	6.02 [153.1]	1.58 [40.2]	2.26 [103.8]	0.60 [7.1]	2.11 [3.1]	1.09	0.79	5.83 [1.7]
	46-	25	6.42 [145.3]	0.46 [8.3]	1.67 [97.9]	0.96 [10.7]	2.54 [2.5]	1.29	0.25	4.16 [0.83]
Women										
	Overall	48	5.98 [78.4]	0.98 [36.9]	1.88 [91.38]	2.33 [16.1]	2.08 [2.0]	1.29	1.0	14.6 [4.2]
	18-35	24	5.41 [75.5]	1.42 [45.7]	2.13 [86.0]	3.0 [19.6]	2.0 [1.9]	1.75	1.46	6.3 [2.1]
	36-45	13	6.69 [100.0]	0.77 [30.8]	2.08 [106.2]	2.77 [16.2]	2.31 [1.6]	1.15	0.92	6.3 [2.1]
	46-	11	6.36 [59.0]	0.27 [25.0]	1.09 [82.0]	0.36 [8.0]	2.0 [2.4]	0.46	0.1	2.1 [0.0]

Table 3- Beverages frequency of consumption, from healthy blood donors living in the Valencian Community, in times per week and average quantity of amount consumed each time. Data collected from 168 questionnaires are shown in overall and also split by sex and age.

	Age range	Number of cases	Days of beer consumption /week [Avg. quantity in mL]	Days of wine consumption / week [Avg. quantity in mL]	Days of coffee consumption / week [Avg. quantity coffees/day]	Days of fruit juice consumption/ week [Avg. quantity in mL]	Days of infusion consumption/ week [Avg. quantity infusions/day]
All data		168	2.31 [303.6]	1.22 [100.6]	5.10 [1.6]	2.98 [164.1]	1.26 [0.43]
Men							
	Overall	120	2.85 [348.3]	1.38 [114.6]	5.02 [1.5]	3.31 [182.7]	1.07 [0.4]
	18-35	42	2.36 [361.9]	0.71 [86.9]	4.95 [1.5]	3.43 [198.6]	1.07 [0.5]
	36-45	53	3.13 [292.5]	1.34 [116.0]	4.89 [1.5]	3.51 [176.9]	0.87 [0.3]
	46-	25	2.92 [450.0]	2.38 [147.9]	5.33 [1.4]	2.67 [166.7]	1.54 [0.5]
Women							
	Overall	48	0.96 [186.9]	0.83 [63.3]	5.29 [1.7]	2.17 [115.0]	1.73 [0.5]
	18-35	24	0.54 [173.9]	0.33 [39.1]	4.71 [1.5]	3.33 [183.8]	2.17 [0.6]
	36-45	13	1.92 [238.5]	1.85 [129.2]	6.46 [1.9]	0.08 [15.4]	1.31 [0.3]
	46-	11	0.73 [150.0]	0.73 [40.0]	5.18 [1.9]	2.10 [100.0]	1.27 [0.5]

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Table 4- Frequency of consumption in times per week of different products described as contaminated with OTA and typical in the Mediterranean diet. In some cases the amount consumed each time is also shown. Data collected from 168 questionnaires are shown in overall and also split by sex and age.

	Age range	Number of cases	Days of chocolate consumption / week [Avg. quantity in grams]	Days of dried fruits consumption / week	Days of cheese consumption / week [Avg. quantity in grams]	Days of spices consumption/ week	Days of olive consumption/ week [Avg. quantity of olives]
All data		168	1.69 [13.7]	1.78	3.88 [59.4]	1.86	1.68 [4.7]
Men							
	Overall	120	1.83 [14.3]	1.81	3.74 [60.5]	1.97	1.93 [5.2]
	18-35	42	2.14 [19.5]	2.43	4.29 [72.0]	2.38	1.95 [5.1]
	36-45	53	1.75 [11.1]	1.25	3.62 [56.0]	1.83	1.70 [4.9]
	46-	25	1.50 [12.5]	1.96	3.00 [48.5]	1.58	2.38 [6.0]
Women							
	Overall	48	1.33 [11.9]	1.67	4.25 [56.6]	1.60	1.04 [3.4]
	18-35	24	1.58 [16.9]	1.46	4.50 [57.8]	1.58	1.08 [3.3]
	36-45	13	1.08 [8.4]	1.38	4.00 [42.3]	1.77	1.08 [3.5]
	46-	11	1.09 [5.4]	2.45	4.00 [70.9]	1.45	0.91 [3.5]

Table 5- Ochratoxin A levels found in blood plasma and body mass index from 168 healthy donors. Cases are split by sex and age.

	Age range	Number of cases	B.M.I ^a	OTA $\mu\text{g/L}$ plasma \pm S.D. ^b	Range OTA $\mu\text{g/L}$ plasma
All data		168	25.92	1.09 ± 0.95	0.15 - 5.71
Men					
	Overall	120	26.2	1.14 ± 0.97	0.15 - 5.71
	18-35	42	25.13	1.13 ± 0.97	0.15 - 5.71
	36-45	53	26.43	1.35 ± 1.11	0.22 - 5.10
	46-	25	27.5	0.76 ± 0.43	0.21 - 2.00
Women					
	Overall	48	25.24	0.98 ± 0.92	0.26 - 5.62
	18-35	24	25.43	0.81 ± 0.62	0.26 - 2.45
	36-45	13	24.32	1.38 ± 1.41	0.37 - 5.62
	46-	11	25.93	0.92 ± 0.74	0.37 - 2.89

a: Body Mass Index

b: Standard Deviation

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Table 6. Correlation coefficients (Spearman rank) and correlation probabilities (Gamma correlations) between food consumption and blood plasma levels of ochratoxin A. Only food items and habits data for which statistically significant correlation coefficients ($P<0.05$) were obtained in one or more of the populations are shown.

Variables	Overall population		Men		Women	
	Spearman rank correlation test	<i>Gamma</i> correlation statistic	Spearman rank correlation test	<i>Gamma</i> correlation statistic	Spearman rank correlation test	<i>Gamma</i> correlation statistic
Beef consumption	0.199^a	0.181	0.173	0.155	0.214	0.205
Turkey consumption	0.163	0.183	0.184	0.220	0.237	0.249
Cold meat consumption	0.220	0.182	0.287	0.235	-0.103	-0.084
Bread consumption	0.211	0.149	0.206	0.146	0.150	0.046
Wine consumption	-0.052	-0.047	-0.161	-0.148	0.332	0.405
Cheese consumption	0.114	0.082	0.043	0.031	0.336	0.237
Habits						
Meals outside home	0.202	0.240	0.179	0.208	0.237	0.314

^a Figures given in bold indicate that the correlation is statistically significant ($P<0.05$)