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Analysis of Ochratoxin A in Foods Consumed by Inhabitants from an Area with Balkan Endemic Nephropathy: A 1 Month Follow-up Study

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In the 1950s, a series of publications from Bulgaria, Yugoslavia, and Romania locally described a kidney disease called Balkan Endemic Nephropathy (BEN). In Bulgaria, the exposure of populations to ochratoxin A (OTA) was supported by analysis of individual food items demonstrating a higher prevalence and higher levels of OTA in food from the high-incidence areas of BEN. In this work, food consumption from a series of individuals from two villages of the BEN area during 1 month was followed using the duplicate diet method. Meals consumed by volunteers from both villages showed uneven OTA contents, spreading from below the limit of quantification (<0.07 μg/kg) to 2.6 μg/kg. The average weekly intake of OTA varies from 1.86 to 92.7 ng/kg of body weight. Some of these levels approach the provisional tolerable weekly intake (PTWI) established by the JECFA at 100 ng/kg of body weight. These results confirm previous studies performed in the same area and demonstrate the high exposure of this population to OTA, thus strengthening the hypothesis of the involvement of this mycotoxin in BEN etiology.

KEYWORDS: Ochratoxin A; Balkan Endemic Nephropathy (BEN); food contamination; dietary intake

INTRODUCTION

A series of publications in the 1950s described a kidney disease in Bulgaria, the former Yugoslavia, and Romania that became known as Balkan Endemic Nephropathy (BEN). This disease was qualified by World Health Organization experts as "...progressive and very gradually developing renal failure with insidious onset...The last stage shows marked fibrosis...". BEN is characterized by tubular degeneration, interstitial fibrosis, and hyalinization of glomeruli accompanied by enzymuria, impaired renal function without degenerative nephrotoxicity. In villages with hyperendemic incidence in Bulgaria, the age-adjusted incidence rates of BEN were $506/10^5$ and $315/10^5$ for females and males, respectively, between 1965 and 1974 (*1*). Similar high incidence rates continue to be recorded. In Kaniza (Croatia), a hyperendemic village for these diseases, an incidence rate of $503/10^5$ was found for the period 1975−1979, and in the next three five-year periods, it ranged between 212 and $246/10^5$ (*2*).

Later, an association between BEN and tumors of the kidney pelvis and ureter was recognized (*1*, *3*−*8*), so that the problem of BEN became not only a nephrological but also an oncological one. Although a few cases of BEN have been diagnosed before the age of 50 years, most occur in the range of 50−60 years, and tumors may be detected even later (*1*). The most affected organs are the renal pelvis and ureter, with combined age-adjusted incidence for these two organs of $74.2/10^5$ and $43.5/10^5$ for females and males, respectively, in Bulgaria (*1*). These values are much higher than those from Trieste, Italy, where rates are already among the highest in the world (2.3 and $5.6/10^5$ for females and males). In the endemic area of the Slavonski Brod district in Croatia, the incidence of urothelial tumors during 1974−1989 reached the extremely high rate of $66.4/10^4$ (*9*). There may also be an association with increased urinary bladder cancer incidence, although many confounding factors may interfere in the analysis of data for this organ.

Several hypotheses concerning the etiology of these diseases have been investigated. The familial clustering of the disease suggests the involvement of predisposing genes. However,

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studies on migrants (*5*, *10*) strongly implicate environmental factors. Numerous studies on the role of environmental and host factors have been carried out to elucidate the etiology of both diseases. Such factors include heavy metals and minerals; bacteria, leptospira, and viruses; fungal toxins and, recently, Pliocene lignites reviewed by Pfohl-Leszkowicz et al. (*11*). Among these hypotheses, the role of fungal toxins has gained importance. In 1972, on the basis of a series of epidemiological observations, Akhmeteli (*12*) suggested that fungal toxins could be involved in the etiology of BEN. Krogh (*13*), in view of the similarities between BEN and ochratoxin A (OTA)-induced porcine nephropathy, suggested that this toxin may be involved in the etiology of BEN. In addition, OTA is carcinogenic to mice and rats (*14*−*16*), affecting mainly the kidney but also the bladder.

Surveys of foodstuffs in the endemic area of the former Yugoslavia demonstrated that 8−12% of the cereals were contaminated with OTA (*17*−*20*). Because OTA binds to serum albumin (*21*), the entire food chain including meat can become contaminated, as demonstrated by Pepeljnjack and Blazević (*22*), who reported the presence of OTA in a variety of smoked meat products.

A similar study of food contamination conducted in Bulgaria demonstrated that a higher percentage of the staple food (maize and beans) was contaminated by OTA in the endemic area than in the nonendemic area (*23*−*25*). Some of these samples were also contaminated by citrinin (*11*), and in a recent study, Vrabcheva et al. (*26*) found that in the BEN endemic area wheat samples could also be contaminated by OTA and at higher levels than in the control nonendemic region.

Previous studies in the Balkans concentrated on the analysis of OTA in staple foods. In these studies, it was necessary to correlate these concentrations in foods with a detailed individual consumption to estimate the OTA intake. In the present study, we wanted to confirm the exposure of a population to OTA in the Vratza district in Bulgaria and follow up the frequency of consumption of OTA-contaminated food by some selected families in the BEN area and the intake level during 1 month. For this, we used the duplicate-diet method already developed by Gilbert et al. (*27*).

MATERIALS AND METHODS

Food Collection. Nineteen healthy 20−30-year-old volunteers were selected from two villages considered as a high risk area for BEN disease: 7 from Gorno Peshtene (GP) and 9 from Beli Izvor (BI) located in Vratza District, northwestern Bulgaria. All volunteers signed a written consent form after the purpose of the study had been explained in the Bulgarian language. All of the volunteers had clinically normal blood biochemistry, hematology, and urine analysis and exhibited negative results to hepatitis B surface antibodies. None of them displayed renal disability; volunteers with a history of hepatic, renal, or metabolic disorders or cardiovascular or gastrointestinal diseases were excluded. Also excluded were people who had undergone any surgical operation on the digestive tract other than an appendectomy or who had a history of alcohol or drug abuse. Participants declared that they would consume their normal diet for the duration (1 month) of the study.

Participants were asked to prepare duplicates of each daily portion of cooked meals and to store them in a refrigerator until collection. They were also asked to record all ingredients used for the preparation of each meal. These cooked food samples were collected during the last quarter of 1998, once a week, weighed, and homogenized by mixing in a blender, and weekly aliquots representative of the daily portions were separated and stored at −20 °C. Thus, for each volunteer, four samples of weekly mixed food representing 4 weeks of meals were collected. For beverages, when people consumed the same drinks purchased from the same shop, people were asked only to record the volume drunk, and the beverage was purchased from the shop for analysis. Finally, samples were recorded as followed: GP or BI for the village of origin followed by figures from 01 to 09 (for GP) and from 01 to 12 (for BI), representing volunteers, and followed by figures 1−4 corresponding to the week of collection of food samples: for instance, BI 02 4 stands for volunteer 2, from Beli Izvor, representative aliquot of samples collected during week 4.

Method of Analysis for OTA. *Reagents.* All solvents and reagents were of analytical or HPLC grade. Water was of HPLC quality generated by a Milli-Q system (Millipore). Ochratoxin A was obtained from Sigma-Aldrich. A stock standard solution of OTA was made in methanol and the concentration measured by spectrophotometry and calculated by using a molar extinction coefficient ϵ of 550 m^2/mol at 330 nm when diluted in pure methanol. OTA working solutions were daily prepared by dilution of the stock OTA solution in 10% acetonitrile. Immunoaffinity columns OchraPrep (IAC) were purchased from Rhone Diagnostics Technologies Ltd. Orthophosphoric acid 85%, acetic acid, and boron trifluoride were from Merck (Paris, France).

HPLC Equipment and Chromatographic Conditions. The OTA analysis of food and beverage samples was carried out using a 655A-12 chromatography pump, an F-1050 fluorescence spectrophotometer (excitation and emission wavelengths being set at 335 and 465 nm, respectively), and a 655A-40 autosampler, all modules being from Merck (Paris, France). The HPLC column was a 100 RP-18 Lichrospher (250 × 4 mm i.d.) from Merck (Paris, France). The composition of the mobile phase was acetonitrile/water/acetic acid (500:500:20) delivered at a flow rate of 0.9 mL/min, and injection volumes were 50 μL.

In-house Characterization of the Analytical Protocol for OTA Determination. The analytical protocol for OTA determination, adapted from a previously developed protocol for analysis of pig kidneys (*28*), was in-house characterized in food matrices toward the following criteria: linearity, accuracy (by determination of the recovery factor), and limit of quantification. Linearity was determined from a stock solution of 10 μg of OTA/mL in methanol, working solutions (at 0.1, 0.5, 1, 2.5, and 5 ng OTA/mL) were prepared just before use by dilution in 10% acetonitrile solution. Calculation of the limit of quantification was based on a signal-to-noise ratio of 6:1 at the retention time of OTA (i.e., 9.5 min) when a noncontaminated sample of food mixture was analyzed. Accuracy was estimated by determining the recovery factor on spiked samples, that is, seven and two samples of a mixture of typical French meals spiked at 4 and 2 μg of OTA/kg, respectively. In addition, several Bulgarian food mixtures (samples from series BI 02, BI 03, and BI 05) were also spiked at 2, 0.15, and 0.10 μg of OTA/kg.

Extraction of Samples of Solid Food and Beverages. A 10 g portion of a finely ground sample of solid food was extracted with 100 mL of chloroform acidified with 1.5 mL of orthophosphoric acid with the help of an Ultra-Turrax set on high speed for 1 min. The mixture was then centrifuged at 3000*g* for 15 min. The chloroform extract was filtered through Whatman no. 4 filter paper and quantitatively transferred to a separating funnel. An equal volume of 0.5 M sodium hydrogenocarbonate was added to the extract and the mixture vigorously shaken. An aliquot (20 mL) of the aqueous phase (upper layer) was ready to be loaded onto the IAC. For extraction of samples of red and white wines and of Boza (a typical Bulgarian nonfermented beverage obtained from barley), 5 mL of sample was added to 40 mL of a 0.1 M hydrochloric acid/0.2 M magnesium chloride (v/v) solution and thoroughly mixed in a Vortex-like stirrer for 1 min. After the addition of 10 mL of chloroform and intensive mixing for 2−3 min, the mixture was spun at 3000*g* for 30 min. The clear organic phase (chloroform) at the bottom of the tube was carefully withdrawn by Pasteur pipet and transferred to a pear-shape flask. The extraction of the aqueous layer was repeated with another 10 mL of chloroform. The combined chloroformic extracts were evaporated to dryness at 60 °C under a gentle stream of N_2. The residue was resuspended in 1 mL of methanol and then in 50 mL of TRIS-HCl (12.11 g of TRIS in 500 mL of distilled water buffered at pH 7.5) before loading of the whole extract onto the IAC. For the extraction of other beverages (liquid coffee, tea, and syrup) 25 mL of sample was added to 25 mL of phosphate-buffered saline solution (0.1 M of KH_2PO_4 + K_2HPO_4, 0.2 g/L KCl, 8 g/L NaCl, pH

7.4) and intensively mixed for 1 min. Ten milliliters from this mixture was ready to be loaded onto the IAC.

Immunoaffinity Cleanup of Samples. Extracts from solid food (20 mL), wine and boza (50 mL), and coffee, tea, or syrup (10 mL) were loaded onto the IAC at a steady flow rate of 2−3 mL/min. After the IAC had been washed with 50 mL of a methanol/water solution (4:96) at a flow rate of ∼5 mL/min, OTA was slowly eluted from the column by passing 2 mL of acidified methanol (methanol/acetic acid, 98:2) by gravity and collected in a sample vial. The column gel was air-dried with the help of a syringe barrel to collect the last drops of eluate. The eluate was then evaporated to nearly dryness at 50 °C under a gentle stream of N_2, and the residue was resuspended in 1 mL of 10% acetonitrile. An aliquot (50 μL) of the final suspension was injected into the HPLC system. The OTA levels of samples were quantified by comparing the height of the sample chromatographic peaks to those of appropriate standard solutions in the range of 0.1−5 ng/mL.

Confirmation of the Presence of OTA. As described by Lerch and Muller (*29*), the confirmation of the presence of OTA in samples first detected at 1 μg of OTA/kg was performed by the methyl ester technique in producing the methyl ester derivative of OTA. Briefly, an aliquot taken from immunoaffinity eluates was dried and dissolved in a methanol solution of boron trifluoride and heated at 60 °C for 15 min. In sample containing OTA, when using the same HPLC chromatographic conditions as for the analysis of OTA, the disappearance of the OTA peak (retention time at 9.5 min) should be observed together with the appearance of the methyl ester peak (retention time of 23.5 min). Quantitative measurement of the methyl ester derivative of OTA is calculated after the calibration curve obtained when the standard solutions of OTA are derivatized in the same way.

Results were not corrected for recovery.

RESULTS

Food Collection. Some typical weekly food components are described hereafter, most of them being homemade unless otherwise specified.

Breakfast. *Raw and cooked meals, containing mainly vegetables or fruits*, consisted of onions; tomatoes, peppers, eggs, and white cheese fried in oil; tomato sauce fried in oil, peppers, and garlic; apples; dried plums; jams from green tomatoes, plums, or strawberries; compote (stewed fruits) of raspberries; peach nectar; and juice of elder.

Cooked dishes containing mainly cereals included boiled corn; Khalva (a paste of ground sesame, sugar, and oil from a shop); baked sunflower seeds; small fried pancakes (made with yeast); small fried rice pancakes; waffles (from a shop), bread; toasted or fried slices of bread (from a shop); and pizza with mushrooms.

Cooked dishes containing meat or fish were fried fish (from a shop); bacon; stewed beans with salami (sausage, from a shop); and fried balls of minced meat (from a shop).

Milk-based dishes included white cheese, white cheese sauce with tomatoes, peppers, and garlic; pastry with white cheese; and milk with honey.

Others: herbal tea with or without honey; coffee with honey; and margarine (from a shop).

Lunch or Dinner. *Raw and cooked meals, containing mainly vegetables or fruits,* consisted of pickles (containing green and ripe tomatoes, carrots, cabbage, red or hot peppers, and cucumbers, alone or in mixture); salad of potatoes; salad of cabbage; salad of kohlrabi (home produced) and beans (home produced) with pork; fried, roasted, or stewed potatoes; stewed cabbage; boiled corn (home produced); peppers (home produced); peppers stuffed with beans; peppers stuffed with rice; fried beans; stewed green beans, fried in oil sauce of tomatoes, peppers, and garlic (and sometimes with hot peppers); vine leaves stuffed with rice; raw onions; stewed rice; stewed potatoes, cabbage, and beans (home produced); raw leeks; soup (of beans, lentils, cabbage, small balls of minced meat, hen, chicken, or pork meat); salad of cabbage, carrot, and parsley (home produced); boiled corn (home produced); fried potatoes (home produced); fried small pumpkins (home produced); apples; dried plums (home produced and dried); raspberry compote (stewed fruits, homemade); and pears (home produced).

Cooked dishes containing mainly cereals included baked sunflower seeds (home produced); pastry with pumpkin; waffles; cream of farina (starch); bread and toasted bread (from a shop and homemade); bread (homemade and from a shop); toasted bread; and tart with biscuits.

Cooked dishes containing meat or fish were soup of vegetables, potatoes (with or without vermicelli, lentils, tripe, hen, chicken, pork, or calf); fried salami (sausage); fried fish; fish in tomato sauce; fish stuffed with nuts in tomatoes sauce; cold stewed pork meat; roasted weaned lamb; chicken (homemade); hen with potatoes or with rice or gruel; musaka (meal of minced meat and potatoes); fried balls of minced meat (from a shop) with tomato sauce; hen (home) gruel; roasted pork (home); roasted pork liver; and roasted weaned lamb.

Milk-based dishes included white cheese; pastry with white cheese (homemade); medlar (rotten); white cheese (home produced); curdled milk (homemade); cocoa cream; yogurt from cow's, goat's, and sheep's milk (homemade).

Others: fried or baked eggs; spices.

Drinks included cherry syrup; apricot nectar; red or white wine (homemade); grape brandy; coffee; chocolates; and herbal tea (homemade) with honey (home produced).

Between-Meal Snacks consisted of popcorn; baked sunflower seeds; fish in tomato sauce (from a shop); fried potatoes; compote of peaches, or strawberries, or stewed fruits; custard (cream of burnt sugar); tart of biscuits; pears; apples; chocolate; coffee; red wine.

No attempt was made to present the variety of food by volunteer; however, when the information obtained was analyzed, there was a wide difference in food variety among the different participants. The same was also observed for food intake (see **Tables 1** and **2**) which, on weekly basis varied by a factor of up to 11-fold (BI 02 week 1 versus BI 06 week 1). Some families had a wide variety of food and a reasonably high consumption (**Tables 1** and **2**, column 2; volunteers GP 02, 04, 05, and 06 and volunteer BI 02), whereas others had a very low food consumption (**Tables 1** and **2**, column 2) of a repetitive small variety of food (volunteer BI 06). The average amount of food weekly consumed by participants from Gorno Peshtene ranged from 3.7 to 6.9 kg depending on people, whereas for Beli Izvor the average amount of food (1.3−5.8 kg) greatly differed from one volunteer to another. In this latter village, the lowest amount of food corresponded to that consumed by a woman from an economically poor Gypsy community. Globally, the meal composition is rather poor when compared to European standards. These foods contain generally a lot of homemade products which, in view of poor storage conditions, may be contaminated by OTA. The diet of participant BI 06 came mainly from the local shop.

Characterization of the Method of Analysis for OTA Determination. The calibration curve was established in the range of 0.1−5 ng of OTA/mL ($r = 0.9999$). Limits of quantification were found to be 0.07 and 0.10 μg of OTA/kg for food and beverages, respectively. Recovery ranges obtained in analyzing spiked samples of food mixture were 68−81, 61−87, 73−107, and 50−98% for spiking levels of 4, 2, 0.15, and 0.10 μg of OTA/kg, respectively. Recovery trials were also done for beverages spiked at 2 and 1 μg of OTA/kg and were found

Table 1. Food and Ochratoxin A (OTA) Consumption by Volunteers from the Village of Gorno Peshtene (GP) of the Vratza District (Bulgaria)[a]

volunteer/ sample no.	wt of diet (g)	OTA level in diet (μg/kg)	OTA consumed (ng)
GP 01 1	5300	0.09	477
GP 01 2	3900	<LQ	<LQ
GP 01 3	3630	<LQ	<LQ
GP 01 4	2000	0.21	420
mean level	3707	0.07	224
GP 02 1	6250	0.14	875
GP 02 2	6460	0.12	775
GP 02 3	6300	<LQ	<LQ
GP 02 4	6200	0.07	434
mean level	6302	0.08	521
GP 03 1	3930	0.11	432
GP 03 2	5180	0.07	362
GP 03 3	4950	<LQ	ND
GP 03 4	6400	<LQ	<LQ
mean level	5115	0.04	198
GP 04 1	7950	0.14	1113
GP 04 2	5710	0.11	628
GP 04 3	6750	<LQ	<LQ
GP 04 4	7350	0.08	588
mean level	6940	0.08	582
GP 05 1	7000	<LQ	<LQ
GP 05 2	6800	<LQ	<LQ
GP 05 3	6100	<LQ	<LQ
GP 05 4	6200	0.25	1550
mean level	6525	0.06	387
GP 06 1	4400	<LQ	ND
GP 06 2	7350	<LQ	<LQ
GP 06 3	5600	0.07	392
GP 06 4	6050	<LQ	ND
mean level	5850	0.02	98
GP 09 1	5500	<LQ	<LQ
GP 09 2	3900	<LQ	<LQ
GP 09 3	5800	0.17	986
GP 09 4	3280	0.15	492
mean level	4620	0.08	369

[a] GP corresponds to the village of origin, followed by numbers to identify the patient (01–09) and by numbers 1–4 corresponding to the week of collection of food samples.

at 64−79% for wine, at 68−87% for coffee and tea, and at only 34% for syrup. In addition, recoveries for Boza and sunflower spiked at 2 μg of OTA/kg were found at 75 and 39%, respectively. Taking into account this large variation depending on the type of matrix, none of the results were corrected for recovery.

OTA Contamination of Food and Beverages. Meals consumed by volunteers in the GP village (**Table 1**) were generally contaminated by low levels of OTA: 50% of ingested foods were below the limit of OTA quantification (i.e., <0.07 μg of OTA/kg), and the highest levels of contamination were found in the mixed-food samples GP 05 4 at 0.25 μg of OTA/kg and GP 01 4 at 0.21 μg of OTA/kg. The highest discrepancy in the weekly amount of OTA ingested by GP volunteers was noticed in GP 05, who ingested 1550 ng of OTA in week 4, although he was not exposed to OTA above the limit of quantification levels the other 3 weeks. Unfortunately, we cannot provide an explanation for this difference because the detailed food composition from this volunteer was missing. Sunflower grains and beverages (wine) ingested by volunteers from GP village were not contaminated by OTA.

Table 2. Food and Ochratoxin A (OTA) Consumption by Volunteers from the Village of Beli Izvor (BI) of the Vratza District (Bulgaria)[a]

volunteer/ sample no.	wt of diet (g)	OTA level in diet (μg/kg)	OTA consumed (ng)
BI 02 1	8000	0.43	3440
BI 02 2	7850	0.43	3375
BI 02 3	3850	0.09	346
BI 02 4	3550	0.20	710
mean level	5812	0.29	1968
BI 03 1	5050	0.24	1212
BI 03 2	6700	0.24	1608
BI 03 3	5550	0.20	1110
BI 03 4	3400	0.28	952
mean level	5175	0.24	1220
BI 05 1	3800	0.09	342
BI 05 2	5500	0.10	550
BI 05 3	1800	0.09	162
BI 05 4	2550	0.09	229
mean level	3412	0.09	321
BI 06 1	700	<LQ	<LQ
BI 06 2	1100	0.10	110
BI 06 3	1300	<LQ	<LQ
BI 06 4	2300	0.10	230
mean level	1350	0.05	85
BI 07 1	1100	0.82	902
BI 07 2	6100	2.32	14152
BI 07 3	3290	0.30	987
BI 07 4	4750	1.99	9452
mean level	3810	1.36	6373
BI 08 1	2200	1.35	2970
BI 08 2	6950	1.52	10564
BI 08 3	3450	0.27	931
BI 08 4	4420	2.60	11492
mean level	4255	1.43	6489
BI 09 1	3600	0.19	684
BI 09 2	7150	0.07	500
BI 09 3	3450	0.09	310
BI 09 4	3950	0.07	276
mean level	4537	0.10	442
BI 10 1	850	<LQ	<LQ
BI 11 1	3000	0.08	240
BI 11 2	4300	<LQ	<LQ
BI 11 3	3600	0.16	576
BI 11 4	3100	0.13	403
mean level	3500	0.09	305
BI 12 1	2550	<LQ	<LQ
BI 12 2	4450	<LQ	<LQ
BI 12 3	3500	<LQ	<LQ
BI 12 4	2700	<LQ	<LQ
mean level	3300	<LQ	<LQ

[a] BI corresponds to the village of origin, followed by numbers to identify the patient (01–09) and by numbers 1–4 corresponding to the week of collection of food samples.

Meals consumed by volunteers in the BI village (**Table 2**) exhibited uneven OTA contents, spreading from below the limit of quantification to 2.60 μg of OTA/kg (sample BI 08 4). The average amounts of OTA ingested per week ranged from below the limit of quantification (BI 12) or close to this (85 ng for BI 06) to 6373 ng (BI 07) or 6489 ng (BI 08). Some volunteers

were clearly more exposed to OTA than others: notably, BI 07 ingested 14152 ng of OTA during week 2 and 9452 ng of OTA during week 4, and BI 08 ingested 10564 ng during week 2 and 11492 ng during week 4. These two participants were from the same family, and the food variety differed only slightly. Another big difference was found in the amount of OTA ingested from one week to another. For instance, BI 02 ingested 10 times the amount of OTA during weeks 1 and 2 compared to week 3 and 5 times the amount when compared with week 4; BI 07 and BI 08 ingested far less OTA in weeks 1 and 3 (around 1000 and 2000 ng of OTA) than in weeks 2 and 4 (OTA amounts in the range of 10000−14000 ng). The composition of food showed that food products susceptible to contamination by OTA were included in their meals of weeks 1 and 2 for BI 02 and in weeks 2 and 4 for BI 07 and BI 08. These food products included a lot of homemade or home-produced pork meat and beans and also food such as chicken meat, bread, pizza, pancakes, (red) peppers, and popcorn (homemade). As for BI 12 and BI 06, who were not or nearly not exposed to OTA, examination of the composition of their foods showed that (i) their mean food intake is very low, especially for BI 06, and (ii) no food products known to be commonly contaminated by OTA were included in their meals. None of the beverages (tea, coffee, wines, boza) contained OTA at detectable levels.

DISCUSSION

Although not validated by collaborative studies on these substrates, the method of analysis used in this study was fully investigated and proved to be reliable and reproducible except for syrup and sunflower seeds, where the low recovery may respectively be due to high sugar and high lipid contents, which interfered with the immunoaffinity cleanup.

No attempt was made to analyze the levels of ochratoxin A in the individual food components. However, from the description of the individual meals, it is possible to trace the potential sources of contamination by this toxin. OTA has already been reported in some of the staple foods from this area [maize and beans (*23*, *24*); wheat (*26*); potatoes (*25*)] and in a number of other food components or cooked food over the world [bread (*30*, *31*); spices (*32*−*34*); milk products (*35*−*37*); coffee (*38*−*41*); and wine (*42*)]. The food purchased commercially appears to be less contaminated that that of volunteer BI 06. For volunteer B 12, levels below the limit of quantification have been constantly detected over the month-long study. The food recorded differed from that of the others by a much greater consumption of meat (chicken, lamb, and pork) and a large consumption of fresh vegetables. An evaluation of the sources of intake of OTA in France demonstrated that the contribution of meat would be only ∼3% of the global intake (*43*). This may explain the low intake of OTA for volunteer B 12. Another volunteer (B 06) had a very low intake of OTA; this person had an extremely low food consumption, mainly of products purchased from stores, which were generally less contaminated than the home-stored products.

The average exposure data obtained over the month demonstrate an exposure to OTA for 15 of the 16 volunteers. These levels reflect the situation for these people in autumn after only a short period of storage of home-produced food. According to the information obtained previously on individual food components not only in this country but also in other Balkan countries, the situation may be much more severe in late spring after 6 months of food storage.

The average weekly intake of OTA varies from 130 to 6489 ng, corresponding to 1.86−92.7 ng/kg of body weight if one considers an average weight of 70 kg for an adult or a daily intake of 0.27−13.2 ng/kg of body weight if one considers a standard 70 kg weight for these adults. The EU study (*44*) reports calculated daily intakes of OTA in European countries (see **Table 3**). For adults, these range from 0.15 in Greece to 2.51 in France. In this study, the highest level was >5 times greater than that from the highest calculated average level in European countries. The provisional tolerable weekly intake (PTWI) established by JECFA is 100 ng/kg of body weight (*45*). In the nine volunteers from Beli Izvor, two (BI 07 and BI 08) are very close to this PTWI (92.7 and 91 ng/kg of body weight/week, respectively) with daily peaks up to 200 ng/kg of body weight. It is important to point out that the food collection for this was performed during autumn/early winter. OTA being considered a mycotoxin that develops during storage, the levels found in the food from some volunteers, although close to the PTWI, are probably much lower than those that would have been found in spring/summer.

Table 3. Daily Intakes of Ochratoxin A Calculated for the European Union (2002)

country	intake of OTA (ng/kg of body wt/day)	
	adults	children
Spain	1.18	
Sweden	1.42	2.03
The Netherlands	1.59 (all population)	
	1.26 (consumers only)	
United Kingdom	0.53 (all population)	1.42 (all population)
	0.91 (consumers 16–64 years)	3.55 (1.5–4.5 years)
Norway	0.97 (1.04 men and 0.88 women)	
Portugal	0.81	
Greece	0.15 (all population)	
	0.13 (urban)	
	0.16 (semiurban)	
	0.23 (rural)	
Italy	1.13 (all population)	
	3.52 (consumers only)	
France	2.51 (all population)	3.39 (2–14 years)
	2.31 (15–65 years)	
Germany	1.09	1.82 (<14 years)
		3.14 (girls 4–6 years)
Denmark	1.19	
Finland	1.71	

The high levels found in the food of these volunteers from the BEN area, apparently not affected by any renal disease, are in line with the previously published results of OTA contamination found in some of the individual components (*23*, *24*, *26*, *32*). These confirm the extremely high exposure of the population from this BEN area to OTA and potential involvement of this toxin in this disease. Even more than 15 years after the first reports, the situation seems not to have improved. High OTA food contents are still found, which results in high OTA blood contents (*46*).

In a similar study performed in the United Kingdom, Gilbert et al. (*27*) reported monthly average intakes of OTA in the range of 144−1993 ng of OTA (mean, 487; median, 346 ng); thus, for an average weight of 70 kg per person, the weekly intake would be 2−28 ng/kg of body weight. These values are higher than those calculated in the EU assessment of the dietary intake of ochratoxin A program. This can easily be explained by the method used in this program, based on estimation of food consumption and food contamination, whereas the study by Gilbert et al. (*27*) relied on actual analysis of food taking into account all elements contaminated. Still, the highest weekly intake measured in this study is 3.5 times lower than the PTWI proposed by JECFA. In the present study it can reach 2 times

the PTWI. The Balkan countries will become in the future members of the EU, and all efforts should be made by the EU to improve the health status of their populations to those of the EU countries.

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