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Quantitation of Aristolochic Acids in Corn, Wheat Grain, and Soil Samples Collected in Serbia: Identifying a Novel Exposure Pathway in the Etiology of Balkan Endemic Nephropathy

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Supporting Information

ABSTRACT: While to date investigations provided convincing evidence on the role of aristolochic acids (AAs) in the etiology of Balkan endemic nephropathy (BEN) and upper urothelial cancer (UUC), the exposure pathways by which AAs enter human bodies to cause BEN and UUC remain obscure. The goal of this study is to test the hypothesis that environmental pollution by AAs and root uptake of AAs in the polluted soil may be one of the pathways by which AAs enter the human food chain. The hypothesis driving this study was that the decay of *Aristolochia clematitis* L., a AA-containing herbaceous plant that is found growing widespread in the endemic regions, could release free AAs to the soil, which could be taken up by food crops growing nearby, thereby transferring this potent human nephrotoxin and carcinogen into their edible parts. Using the highly sensitive and selective high-performance liquid chromatography coupled with fluorescence detection method, we identified and quantitated in this study for the first time AAs in corn, wheat grain, and soil samples collected from the endemic village Kutles in Serbia. Our results provide the first direct evidence that food crops and soil in the Balkans are contaminated with AAs. It is possible that the presence of AAs in edible parts of crops originating from the AA-contaminated soil could be one of the major pathways by which humans become exposed to AAs.

KEYWORDS: *Balkan endemic nephropathy, upper urothelial cancer, aristolochic acid nephropathy, aristolochic acids, exposure pathways, root uptake*

INTRODUCTION

Aristolochic acids (AAs; Figure 1) are a group of nitrophenanthrene carboxylic acids found in the herbal genus *Aristolochia* and are classified by the International Agency for Research on Cancer (IARC) as a Group 1 carcinogen.[1−5] Although AAs are banned from use in many countries, misuse of AA-containing herbs occurs.[5−10] For example, the misuse of AA-containing herbs in the preparation of slimming drugs has caused numerous end-stage kidney diseases in Belgian women that participated in a slimming regime in the 1990s.[5,8−10] Accumulating data also indicate AAs as the causative agent of Balkan endemic nephropathy (BEN),[10−15] a chronic kidney disease affecting numerous farmers living in the rural area of the Balkan Peninsula.[16] It has been argued that the problem is caused by the commingling of the fruits of *Aristolochia clematitis* L. with commercial grains.[17] However, the pathways by which AAs enter the human food chain to cause BEN has remained obscure.[3,16,18]

Recently, it was disclosed that *A. clematitis* L., an AA-containing herbaceous plant, is widespread in some areas of the endemic region.[13,19,20] Despite the identification of AAs in the soil samples that has yet to be reported, it is postulated that AAs may have entered the environment from the decay of *A. clematitis* L. Furthermore, we discovered that food crops grown in AA-contaminated environments would uptake and accumulate AAs in the edible parts of the plant.[13,21] We thus proposed root uptake of AAs from the environment may be one of the exposure pathways by which the nephrotoxic AAs enter our food chain.

In this study, we try to confirm root uptake as one of the pathways by which AAs enter human bodies to cause BEN by analyzing soil, corn, and wheat grain samples collected from a well-known BEN-endemic village (Kutles; latitude, 43.140; longitude, 21.862) in Serbia. Using our recently developed high-performance liquid chromatography coupled with fluorescence detection (HPLC−FLD) method, we could detect aristolactams, the fluorescent nitroreduction products of AAs (Figure 1),[22] which allowed us to test for the presence of AAs in wheat grain and soil samples from where the wheat was grown. Further, we have conducted a surveillance analysis of AAs in wheat grain and corn seed samples collected by farmers

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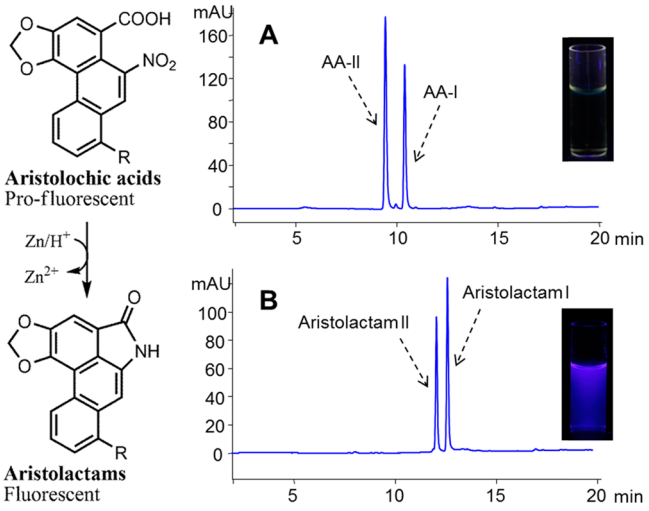

Figure 1. Nitroreduction of AAs (AA-I, R = OCH_3; AA-II, R = H) produce fluorophoric arstiolactams for their highly sensitive determination by HPLC−FLD. Depicted in panels A and B are typical chromatograms obtained from HPLC−UV analysis of the AAs (A) before and (B) after reduction with Zn/H^+. Efficient formation of aristolactams from nitroreduction of AAs was observed.

of the same village. The results from our studies revealed, for the first time, that soil and food crops in an endemic village of Serbia were contaminated with AAs. With significant evidence demonstrating AAs being a strong human carcinogen and potent nephrotoxin,[23−28] it is expected that the finding in this study will alert regulatory agencies of the potential existence of a new contaminant, AAs, in both soil and food crops.

MATERIALS AND METHODS

Caution. AAs are carcinogenic and nephrotoxic to humans and should be handled with caution.

Chemicals and Materials. All chemicals and reagents used were of the highest purity available and were used without further purification. AAs (a mixture of AA-I and AA-II, 1:1) were purchased from Acros (Morris Plains, NJ). HPLC-grade methanol and acetonitrile were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Deionized water was further purified by a Milli-Q Ultrapure water purification system (Millipore, Billerica, MA) and was used in all experiments. C18 solid-phase extraction (SPE) columns packed with 500 mg of sorbents were obtained from Grace (Deerfield, IL).

Instrumentation. HPLC−FLD analysis was performed on an Agilent 1200 HPLC system coupled with a fluorescence detector (Palo Alto, CA). Ultraperformance liquid chromatography coupled with tandem mass spectrometry (UPLC−MS/MS) detection was performed on a Waters UPLC coupled with a Waters Xevo G2 Q-Tof mass spectrometer (Milford, MA). A standard electrospray interface operating at positive ionization mode was used for the UPLC−MS/MS analysis.

Sample Collection. Wheat grain and soil samples were collected manually from an endemic village (village, Kutles; latitude, 43.140; longitude, 21.862; and elevation, 211 m) in Serbia. The study materials, wheat grain (n = 15), were collected from wheat fields with *A. clematitis* L. growing on them (one plant per square meter on average) at the time of sample collection (June, 2015). At the same time of the wheat sample collection, the corresponding soil (n = 15) samples in the immediate surroundings were also collected for analysis. The negative control materials, wheat (n = 15) and the corresponding soil (n = 15) samples, were collected randomly in the wheat field of the same village but from the farmland with no *A. clematitis* L. observed at the time of collection. Another set of wheat grain (n = 70) and corn grain (n = 70) samples for surveillance of AAs were collected from 70 families in the same village. Control soil (n = 5) and wheat (n = 5) samples were also collected from a local in Hong Kong, a non-BEN area. Voucher specimens of all studied samples were collected and stored in a −80 °C freezer for reference.

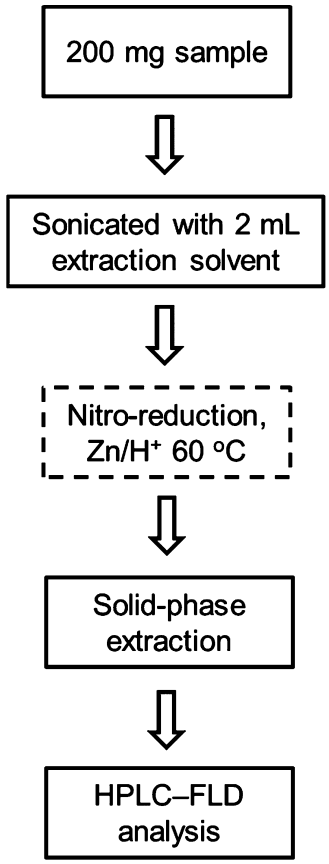

Figure 2. Schematic diagram showing HPLC−FLD analyses of the collected samples. The analysis of the sample extract without the nitroreduction step gave a concentration of naturally occurring aristolactam concentrations, while the analysis of the sample extract after Zn/H^+ treatment gave the total aristolactams, i.e., from both naturally occurring aristolactams and nitroreduction of AAs. The concertation of AAs in the samples were calculated by subtracting the total aristolactam concentration from that of the naturally occurring aristolactams.

Table 1. Calibration Parameters and MDLs of the Developed HPLC−FLD Method for AA Analysis

	AA-I	AA-II
linear range (ng/mL)	20−1200	20−1200
slope	1.453	1.769
intercept	−8.484	−8.664
R^2	0.9987	0.9991
MDL (pg/injection)	33	31
MDL (ng/g)[a]	3.3	3.1

[a]The MDL is the sample extract based on 200 mg of sample.

Sample Preparation. All of the collected samples were dried under the sun and ground to powder using a mortar and pestle. Approximately 200 mg of the samples was accurately weighted and extracted using 2 mL of the extraction solvent (70:25:5, v/v/v, methanol/water/acetic acid). After sonication at 50 °C for 1 h, the samples were treated by Zn/H^+ (at 60 °C for 15 min) to reduce the non-fluorescing AAs to fluorescing aristolactams (Figure 1). The solution was then cleaned up and enriched by SPE as described previously.[21] The purified sample solution was then dried under nitrogen, and the residual was redissolved in 100 μL of methanol before being analyzed by HPLC−FLD. Using a similar procedure, but without the

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Table 2. Validation of the HPLC−FLD Method for the Analysis of Wheat, Corn Grain, and Soil Samples

			precision		accuracy	
		concentration added (ng/g)	intraday (% RSD)[a]	interday (% RSD)[a]	concentration found (ng/g)[b]	% error
wheat grain	AA-I	81	6.5	8.3	74 ± 14	8.64
		812	7.1	9.2	732 ± 34	9.85
		4061	4.6	5.4	3841 ± 174	5.41
	AA-II	89	5.3	6.0	81 ± 16	8.99
		889	7.7	10.5	802 ± 80	9.79
		4445	6.4	8.6	4107 ± 227	7.60
corn grain	AA-I	81	8.2	8.9	87 ± 10	7.41
		812	5.2	6.0	856 ± 68	5.42
		4061	4.0	4.5	3731 ± 172	8.13
	AA-II	89	4.0	5.9	92 ± 21.7	3.37
		889	6.7	8.4	846 ± 38	4.84
		4445	6.3	8.9	4752 ± 252	6.91
soil	AA-I	81	7.2	7.4	88 ± 13	8.64
		812	5.9	8.2	774 ± 44	4.68
		4061	3.8	4.4	4385 ± 156	7.98
	AA-II	89	3.4	5.5	83 ± 12	6.74
		889	6.8	7.6	854 ± 79	4.10
		4445	4.8	6.4	4110 ± 298	7.54

[a] $n = 7$. [b] $n = 3$.

Zn/H^+ treatment step, the concentrations of naturally occurring aristolactams in collected samples were also determined (Figure S1 of the Supporting Information and Figure 2).

HPLC−FLD Analysis. A total of 10 μL of the sample extract was injected onto a Grace C18 column (GraceSmart, 150 × 2.1 mm, 5 μm) for chromatographic separation. The HPLC mobile phase consisted of two components: (A) water and (B) acetonitrile, eluted at a flow rate of 300 μL/min. The solvent gradient started from 20% B, programmed to 100% in 15 min, and was held for 5 min before column reconditioning. The HPLC eluate was monitored by a fluorescence detector with the excitation and emission wavelengths set at 393 and 470 nm, respectively. The detector gain of the fluorescence detector was set at 12.

Calibration. Working standard solution mixtures of AA-I and AA-II at five concentrations (AA-I, 20−1200 ng/mL; AA-II, 20−1200 ng/mL) were prepared and treated with Zn/H^+, as described above, to reduced AAs to aristolactams (Figure 1). The Zn/H^+-treated standard solutions were then centrifuged at 14 000 relative centrifugal force (RCF) for 5 min before the supernatant was analyzed by HPLC−FLD using the method described above. Calibrating curves of AA-I and AA-II were established by plotting the peak areas of aristolactam against the concentrations of AAs in the working standards.

Method Validation. The method was validated for accuracy and precision by analyzing pooled negative control soil, wheat, and corn grain that were fortified with AA-I (0.08, 0.81, and 4.06 μg/g) and AA-II (0.09, 0.89, and 4.45 μg/g). The intra- and interday reproducibilities of the method were evaluated by processing and analyzing the samples on the same day ($n = 7$) and in 7 days over 2 weeks, respectively. Using the same samples, the method accuracy was evaluated by processing and analyzing the AA-fortified samples using the method described above. The minimum detection limit (MDL) was estimated as the amount of analyte that generates a signal 3 times the signal-to-noise (S/N) ratio.[29−33]

RESULTS AND DISCUSSION

Method Calibration and Validation. The HPLC−FLD method developed previously for herbal medicine analysis was validated for the captioned food and soil analysis (Figure 2). The calibration curves of AA-I and AA-II are established by plotting the peak area of aristolactams versus the concentration of AAs in the working standards. The coefficients of determination (R^2) of >0.999 for both AA-I and AA-II indicate that the nitroreduction step is highly quantitative, and thus, the

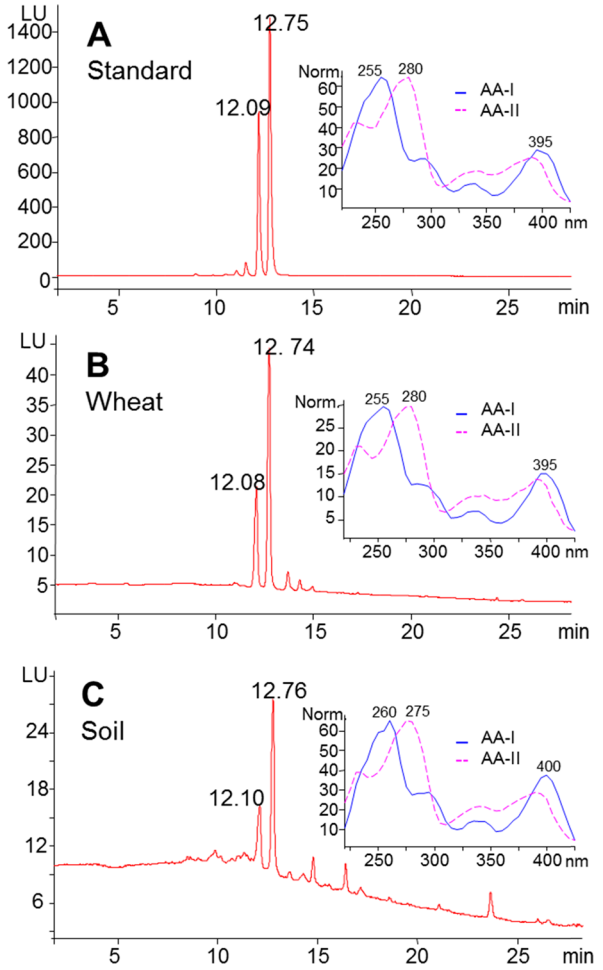

Figure 3. HPLC−FLD analyses of aristolactam I and aristolactam II, the nitroreduction products of AA-I and AA-II, respectively, in a (A) standard solution mixture, (B) typical wheat grain sample, and (C) soil sample collected from endemic village Kutles in Serbia. The nitroreduction products of AA-I and AA-II were eluted at 12.7 and 12.1 min, respectively. Shown in the insets are the fluorescence excitation spectra obtained from analyzing the pooled HPLC fraction containing aristolactams.

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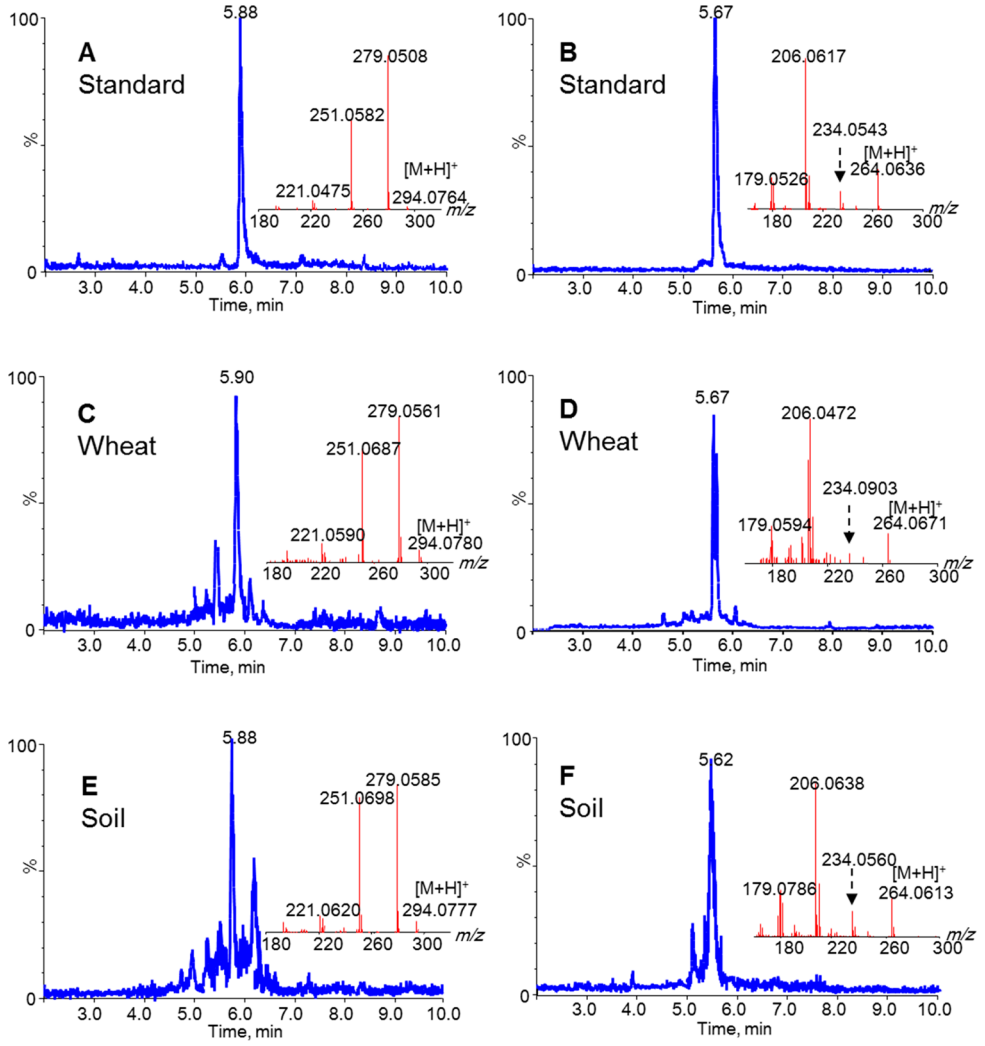

Figure 4. Chromatograms from UPLC−MS/MS analysis of aristolactams derived from nitroreduction of an authentic standard of (A) AA-I and (B) AA-II, (C) AA-I and (D) AA-II in a wheat grain sample collected from Serbia, and (E) AA-I and (F) AA-II in a soil sample collected from Serbia. AA-I and AA-II were eluted at 5.9 and 5.7 min, respectively. Shown in the insets are the MS/MS spectra obtained from analyzing the pooled HPLC fraction containing aristolactams.

detector response for aristolactam is linearly dependent upon the concentration of AAs in the working standards. The MDL defined as the amounts of AA-I and AA-II that generate an analytical signal 3 times the noise level, was 33 and 31 pg/injection, respectively. Because 200 mg of sample was used in each analysis, these MDL values correspond to method detection limits of 3.3 and 3.1 ng of AA-I and AA-II per gram of sample, respectively. Table 1 summarizes the slopes, intercepts, and R^2 of the calibration curves together with the MDLs of the HPLC−FLD method for AA analysis.

The accuracy and reproducibility of the developed HPLC−FLD method for determining AAs in soil, corn, and wheat grain samples were evaluated by spiking to blank samples with the AAs at concentrations of 0.08, 0.81, and 4.06 μg/g (AA-I) and 0.09, 0.89, and 4.45 μg/g (AA-II) (Table 2). Repetitive analysis of the AA-fortified samples on the same day (n = 7) and on 7 different days over 2 weeks revealed intra- and interday precision of relative standard deviation (RSD) less than 8.2 and 10.5%, respectively, for all sample matrices and concentrations. The results indicate that the steps of solvent extraction, Zn/H^+ treatment to reduce AAs to aristolactams, SPE cleanup, and analysis (Figure 2) are highly reproducible.

The accuracy of the method was measured by analyzing the AA-fortified samples (Table 2). Data show that the measured concentrations of both AA-I and AA-II are closely correlated with the spike levels and with no data derivated from the spiked value more than 9.9%. These results demonstrate good recovery (>90%) of both AA-I and AA-II in the extraction and SPE cleanup processes in all of the tested sample matrices.

Identification of AAs in Wheat Grain and Soil Samples. The validated HPLC−FLD method was applied to detect AAs in wheat grain and soil samples collected at the spot that the corresponding wheat was grown. As indicated by the highly similar chromatographic retention times and fluorescence excitation spectra to that of the authentic standards (Figure 3), our analysis revealed the positive identification of both AA-I and AA-II in the collected wheat grain and soil samples. No AAs were detected in both the control soil and wheat samples collected in Hong Kong.

The identification of AAs in the experimental soil and wheat samples was further validated by UPLC−MS/MS analysis. The very similar retention times and MS/MS spectra of aristolactam I and aristolactam II in the collected HPLC fractions to that of the authentic standards unambiguously demonstrated the

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presence of AA-I and AA-II in the collected wheat grain samples (Figure 4). To the best of our knowledge, this is the first time that AA-I and AA-II were detected in wheat grain and soil samples.

Michel et al. recently reported that aristolactams, e.g., aristolactam glucosides, are a key group of compounds in the seeds of *A. clematitis* L.[15] However, as illustrated by HPLC−FLD analysis of the underivatized sample extract of *A. clematitis* L. (Figure S1 of the Supporting Information), our data revealed only a very low concentration of aristolactam I in the seeds of *A. clematitis* L., with no other fluorescing aristolactam-containing compounds, e.g. aristolactam glucosides, being detected at high abundance.

Quantitation of AAs in Wheat Grain and Soil Samples. Analysis of the group of wheat grain samples collected from the fields with *A. clematitis* L. growing revealed positive identification of both AA-I and AA-II in a large proportion of the samples. Specifically, we were able to detect AA-I and AA-II in 12 and 10 of 15 wheat samples (Table 3), respectively. Interestingly, the analysis revealed significantly higher levels of AA-I (91.31 ± 89.02 ng/g) than AA-II (41.01 ± 12.45 ng/g) in the wheat study samples (p = 0.04). This observation is in pretty good agreement with the previous observation that AA-I was detected at a significantly higher concentration than that of AA-II in plant species in the *Aristolochia* genus.[22,34]

Using a similar approach, the concentrations of AAs in the corresponding soil study samples from which the wheat was grown were also quantified (Table 3). Strikingly, we were able to detect both AA-I and AA-II in all of the collected soil samples. Likewise, in the wheat study samples, a significantly higher concentration of AA-I (86.59 ± 27.15 ng/g) than AA-II (25.73 ± 11.46 ng/g) was detected in all of the 15 collected soil study samples (p < 0.001). In particular, AA-I was again detected at 2−5 times higher concentrations than that of AA-II in the collected soil samples. This result is interestingly in reasonable agreement with the observation that, in *A. clematitis* L., AA-I was detected at 7−10 times higher concentrations than that of AA-II.[22,35] The data suggested that distribution of AA-I and AA-II in aerial parts of food crops resembles that of *A. clematitis* L., which serves as the contamination source.

As indicated by the bioaccumulation factor (>1), the study indicated that AAs were efficiently taken up and bioaccumulated in the wheat (Table 3).[36,37] Specifically, the analysis revealed higher levels of AAs in some of the wheat grain than that in the corresponding soil samples from which the wheat were collected, indicating that both AA-I and AA-II were bioaccumulated in wheat grain. Similar phenomena of plant uptake and accumulation were also observed in our previous study using lettuce, spring onion, maize, and cucumber as plant models.[13,21]

Analysis of the control wheat and soil samples originating from wheat fields with no *A. clematitis* L. growing in them at the time of collection identified low levels of AA-I and/or AA-II in some of the wheat grain and soil samples (Table 4). This indicates the possible long-term presence and widespread environmental pollution by AAs in the affected areas. We believe that the soils were contaminated by decomposed material originating from *A. clematitis* L. grown on the same field over previous years and that the AAs from such contaminated soil

Table 4. Concentrations of AA-I and AA-II in Wheat and Soil Control Samples Collected from Endemic Village Kutles in Serbia (Latitude, 43.140; Longitude, 21.862)[a]

	AA-I (ng/g)[b]		AA-II (ng/g)[b]	
	wheat	soil	wheat	soil
KC6	ND[c]	38.1 ± 5.2	ND	16.4 ± 3.7
KC11	30.4 ± 2.3	ND	21.8 ± 2.7	ND
KC12	ND	30.6 ± 4.3	ND	12.9 ± 1.6
KC13	40.3 ± 5.6	49.5 ± 4.3	16.1 ± 2.2	18.4 ± 3.8
KC14	28.8 ± 4.1	ND	16.3 ± 3.2	ND
mean[d]	33.2 ± 1.13	39.4 ± 8.1	18.1 ± 3.9	15.9 ± 1.4

[a]The fields were observed to be with no *A. clematitis* L. growing at the time of sample collection. [b]Processed and analyzed 3 times. [c]ND = not detected. [d]Mean concentrations in samples that showed positive identification of AAs.

Table 3. Concentrations of AA-I and AA-II in Wheat Grain and Soil Samples Collected in Serbia (Village, Kutles; Latitude, 43.140; Longitude, 21.862) from a Wheat Field with *A. clematitis* L. Growing at the Time of Sample Collection

	AA-I (ng/g)[a]			AA-II (ng/g)[a]		
	wheat	soil	bioaccumulation factor	wheat	soil	bioaccumulation factor
K1	NQ[b]	69.8 ± 5.3	NA	ND[c]	18.5 ± 2.2	NA
K2	159.1 ± 9.4	75.2 ± 10.1	2.12	41.4 ± 6.2	24.6 ± 5.4	1.68
K3	275.6 ± 9.1	93.7 ± 12.3	2.94	97.1 ± 4.9	21.4 ± 3.2	4.54
K4	77.3 ± 8.5	128.1 ± 9.5	0.60	33.6 ± 4.5	42.5 ± 5.3	0.79
K5	43.5 ± 5.2	84.6 ± 8.1	0.51	14.3 ± 2.7	37.9 ± 5.5	0.38
K6	ND	93.3 ± 13.2	NA	ND	31.5 ± 3.9	NA
K7	108.3 ± 9.9	74.4 ± 9.5	1.46	35.6 ± 5.1	18.2 ± 3.3	1.96
K8	ND	95.3 ± 10.0	NA	ND	23.6 ± 3.1	NA
K9	44.1 ± 5.7	91.9 ± 8.7	0.48	NQ	21.1 ± 1.8	NA
K10	ND	70.5 ± 8.3	NA	ND	17.7 ± 3.0	NA
K11	92.4 ± 8.2	70.0 ± 5.2	1.32	41.3 ± 5.6	21.6 ± 2.6	1.91
K12	17.7 ± 2.4	88.3 ± 12.3	0.20	NQ	30.4 ± 4.1	NA
K13	61.9 ± 8.8	71.2 ± 6.7	0.87	23.8 ± 4.0	24.5 ± 3.7	0.97
K14	NQ	84.3 ± 10.6	NA	ND	17.8 ± 2.0	NA
K15	33.2 ± 4.1	108.2 ± 7.8	0.28	NQ	34.7 ± 3.9	NA
mean[d]	91.3 ± 89.0	86.6 ± 27.2	1.08	41.0 ± 12.5	25.7 ± 11.5	1.75

[a]Processed and analyzed 3 times. [b]NQ = detected but cannot be quantified. [c]ND = not detected. [d]Mean concentrations in samples that showed positive identification of AAs.

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were taken up by the wheat. This hypothesis was supported by our previous observation that AAs are stable to microbial activities of the soil and are highly persistent in the soil.[21]

Results from HPLC−FLD analysis of wheat grain and soil samples collected from both wheat fields with and without *A. clematitis* L. growing revealed that AA is a widespread environmental and food contaminant in the endemic region in the Balkan areas. To the best of our knowledge, this is the first report demonstrating the detection of AAs in environmental and food samples. It is possible that the decay of the *A. clematitis* L. plant and fruit in the soil discharge AAs to the soil, which were later being taken up and transported into edible food crops to cause BEN.

Surveillance of AAs in Corn and Wheat Grain Samples Collected from a Village in the Endemic Region in Serbia. After AAs were successfully detected in wheat grain and soil samples, the study was extended to investigate the occurrence of AAs in wheat grain (n = 70) and corn grain (n = 70) samples collected by farmers in the village Kutles, a well-known endemic village in Serbia. Surprisingly, we were able to detect both AA-I and AA-II in more than half of the collected wheat samples (Table 5). Specifically, AA-I and AA-II were detected in 59 and 36 of the 70 samples and at concentration ranges of 20.0−1178.9 and 16.4−325.8 ng/g, respectively. Assuming 100 kg of wheat was consumed per capita per annum, these data correspond to a maximum exposure per annum of 117.9 mg of AA-I and 32.6 mg of AA-II. Similarly, AAs were also detected in the corn grain samples collected from the farmers (Table 5).

Table 5. Results of Surveillance of AAs Collected in Corn and Wheat Grain Samples[a]

		AA-I	AA-II
wheat grain[b]	cases of positive identification	59	36
	concentration range (ng/g)	20.0−1178.9	16.4−325.8
	average concentration (ng/g)[c]	152.0	49.6
corn grain[b]	cases of positive identification	36	19
	concentration range (ng/g)	19.7−893.7	16.3−167.0
	average concentration (ng/g)[c]	77.2	30.5

[a]Wheat and corn grain samples were collected in Serbia (village, Kutles; latitude, 43.140; longitude, 21.862), processed, and HPLC−FLD-analyzed as described in the Materials and Methods. [b]n = 70. [c]Mean concentrations in samples that showed positive identification of AAs.

Our results unequivocally demonstrated, for the first time, that AA-I and AA-II are the widespread environmental contaminants in the region existing endemic nephropathy in the Balkan Peninsula. The root uptake of AA-I and AA-II from the environment was identified to be one of the principal pathways by which AAs enter human and very likely animal food chains. We believe that the results from this study have effectively resolved an almost 60-year-old mystery of exposure pathways so important in elucidating the etiology of BEN and/or aristolochic acid nephropathy (AAN), which are now considered as a worldwide problem.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02203.

Chromatograms from HPLC−FLD analysis of underivatized and Zn/H$^+$-treated AAs in (A) authentic standard, (B) seed of *A. clematitis* L., (C) soil, and (D) wheat samples (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

Wan Chan and Nikola M. Pavlović designed the research; Nikola M. Pavlović, Biljana Milosavljević, and Emina N. Kostić collected soil, wheat, and corn grain samples; Weiwei Li and Chi-Kong Chan performed the research; Wan Chan, Nikola M. Pavlović, Weiwei Li, Chi-Kong Chan, Jingjing Liu, Kailin Deng, and Yinan Wang analyzed data; and Wan Chan and Nikola M. Pavlović wrote the paper.

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■ ABBREVIATIONS USED

AA, aristolochic acid; AAN, aristolochic acid nephropathy; BEN, Balkan endemic nephropathy; CHN, Chinese herb nephropathy; HPLC−FLD, high-performance liquid chromatography coupled with fluorescence detection; UPLC−MS/MS, ultraperformance liquid chromatography coupled with tandem mass spectrometry; ND, not detected; NQ, not quantified; UUC, upper urothelial cancer

■ REFERENCES

(1) Stiborová, M.; Frei, E.; Arlt, V. M.; Schmeiser, H. H. Metabolic activation of carcinogenic aristolochic acid, a risk factor for Balkan endemic nephropathy. *Mutat. Res., Rev. Mutat. Res.* **2008**, *658*, 55−67.

(2) Arlt, V. M.; Stiborová, M.; vom Brocke, J.; Simoes, M. L.; Lord, G. M.; Nortier, J. L.; Hollstein, M.; Phillips, D. H.; Schmeiser, H. H. Aristolochic acid mutagenesis: Molecular clues to the aetiology of Balkan endemic nephropathy-associated urothelial cancer. *Carcinogenesis* **2007**, *28*, 2253−2261.

(3) Arlt, V. M.; Stiborova, M.; Schmeiser, H. H. Aristolochic acid as a probable human cancer hazard in herbal remedies: A review. *Mutagenesis* **2002**, *17*, 265−277.

(4) Hashimoto, K.; Higuchi, M.; Makino, B.; Sakakibara, I.; Kubo, M.; Komatsu, Y.; Maruno, M.; Okada, M. Quantitative analysis of aristolochic acids, toxic compounds, contained in some medicinal plants. *J. Ethnopharmacol.* **1999**, *64*, 185−189.

(5) Debelle, F. D.; Vanherweghem, J. L.; Nortier, J. L. Aristolochic acid nephropathy: A worldwide problem. *Kidney Int.* **2008**, *74*, 158−169.

DOI: 10.1021/acs.jafc.6b02203
J. Agric. Food Chem. 2016, 64, 5928−5934

(6) Cosyns, J. P. Aristolochic acid and 'Chinese herbs nephropathy. *Drug Saf.* **2003**, *26*, 33−48.
(7) Wu, K. M.; Farrelly, J. G.; Upton, R.; Chen, J. Complexities of the herbal nomenclature system in traditional Chinese medicine (TCM): Lessons learned from the misuse of *Aristolochia*-related species and the importance of the pharmaceutical name during botanical drug product development. *Phytomedicine* **2007**, *14*, 273−279.
(8) Vanherweghem, J. L. Misuse of herbal remedies: The case of an outbreak of terminal renal failure in Belgium (Chinese herbs nephropathy). *J. Altern. Complementary Med.* **1998**, *4*, 9−13.
(9) Isnard Bagnis, C.; Deray, G.; Baumelou, A.; Le Quintrec, M.; Vanherweghem, J. L. Herbs and the kidney. *Am. J. Kidney Dis.* **2004**, *44*, 1−11.
(10) Vanherweghem, J. L.; Tielemans, C.; Abramowicz, D.; Depierreux, M.; Vanhaelen-Fastre, R.; Vanhaelen, M.; Dratwa, M.; Richard, C.; Vandervelde, D.; Verbeelen, D.; Jadoul, M. Rapidly progressive interstitial renal fibrosis in young women: Association with slimming regimen including Chinese herbs. *Lancet* **1993**, *341*, 387−391.
(11) Grollman, A. P.; Shibutani, S.; Moriya, M.; Miller, F.; Wu, L.; Moll, U.; Suzuki, N.; Fernandes, A.; Rosenquist, T.; Medverec, Z.; Jakovina, K.; Brdar, B.; Slade, N.; Turesky, R. J.; Goodenough, A. K.; Rieger, R.; Vukelic, M.; Jelakovic, B. Aristolochic acid and the etiology of endemic (Balkan) nephropathy. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 12129−12134.
(12) Yun, B. H.; Rosenquist, T. A.; Sidorenko, V.; Iden, C. R.; Chen, C. H.; Pu, Y. S.; Bonala, R.; Johnson, F.; Dickman, K. G.; Grollman, A. P.; Turesky, R. J. Biomonitoring of aristolactam-DNA adducts in human tissues using ultra-performance liquid chromatography/iontrap mass spectrometry. *Chem. Res. Toxicol.* **2012**, *25*, 1119−1131.
(13) Pavlovic, N. M.; Maksimovic, V.; Maksimovic, J. D.; Orem, W. H.; Tatu, C. A.; Lerch, H. E.; Bunnell, J. E.; Kostic, E. N.; Szilagyi, D. N.; Paunescu, V. Possible health impacts of naturally occurring uptake of aristolochic acids by maize and cucumber roots: Links to the etiology of endemic (Balkan) nephropathy. *Environ. Geochem. Health* **2013**, *35*, 215−226.
(14) Gold, L. S.; Slone, T. H. Aristolochic acid, an herbal carcinogen, sold on the Web after FDA alert. *N. Engl. J. Med.* **2003**, *349*, 1576−1577.
(15) Michl, J.; Ingrouille, M. J.; Simmonds, M. S.; Heinrich, M. Naturally occurring aristolochic acid analogues and their toxicities. *Nat. Prod. Rep.* **2014**, *31*, 676−693.
(16) Tatu, C. A.; Orem, W. H.; Finkelman, R. B.; Feder, G. L. The etiology of Balkan endemic nephropathy: Still more questions than answers. *Environ. Health Perspect.* **1998**, *106*, 689−700.
(17) Michl, J.; Kite, G. C.; Wanke, S.; Zierau, O.; Vollmer, G.; Neinhuis, C.; Simmonds, M. S. J.; Heinrich, M. LC-MS- and ^{1}H NMR-Based Metabolomic Analysis and in Vitro Toxicological Assessment of 43 *Aristolochia* Species. *J. Nat. Prod.* **2016**, *79*, 30−37.
(18) Bamias, G.; Boletis, J. Balkan nephropathy: Evolution of our knowledge. *Am. J. Kidney Dis.* **2008**, *52*, 606−616.
(19) Slade, N.; Moll, U. M.; Brdar, B.; Zoric, A.; Jelakovic, B. p53 mutations as finger-prints for aristolochic acid: An environmental carcinogen in endemic (Balkan) nephropathy. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **2009**, *663*, 1−6.
(20) De Broe, M. E. Chinese herbs nephropathy and Balkan endemic nephropathy: Toward a single entity, aristolochic acid nephropathy. *Kidney Int.* **2012**, *81*, 513−515.
(21) Li, W.; Hu, Q.; Chan, W. Uptake and Accumulation of Nephrotoxic and Carcinogenic Aristolochic Acids in Food Crops Grown in *Aristolochia clematitis*-Contaminated Soil and Water. Journal of agricultural and food chemistry. *J. Agric. Food Chem.* **2016**, *64*, 107−112.
(22) Chan, W.; Lee, K. C.; Liu, N.; Cai, Z. A sensitivity enhanced high-performance liquid chromatography fluorescence method for the detection of nephrotoxic and carcinogenic aristolochic acid in herbal medicines. *J. Chromatogr. A* **2007**, *1164*, 113−119.
(23) Priestap, H. A.; Barbieri, M. A. Conversion of aristolochic acid I into aristolic acid by reaction with cysteine and glutathione: Biological implications. *J. Nat. Prod.* **2013**, *76*, 965−968.
(24) Yu, F. Y.; Lin, Y. H.; Su, C. C. A sensitive enzyme-linked immunosorbent assay for detecting carcinogenic aristolochic acid in herbal remedies. *J. Agric. Food Chem.* **2006**, *54*, 2496−2501.
(25) Stiborová, M.; Frei, E.; Wiessler, M.; Schmeiser, H. H. Human enzymes involved in the metabolic activation of carcinogenic aristolochic acids: Evidence for reductive activation by cytochromes P450 1A1 and 1A2. *Chem. Res. Toxicol.* **2001**, *14*, 1128−1137.
(26) Leung, E. M. K.; Chan, W. Comparison of DNA and RNA adduct formation: Significantly higher levels of RNA than DNA modifications in the internal organs of aristolochic acid-dosed rats. *Chem. Res. Toxicol.* **2015**, *28*, 248−255.
(27) Schmeiser, H. H.; Bieler, C. A.; Wiessler, M.; de Strihou, C. V. Y.; Cosyns, J. P. Detection of DNA adducts formed by aristolochic acid in renal tissue from patients with Chinese herbs nephropathy. *Cancer Res.* **1996**, *56*, 2025−2028.
(28) Stiborová, M.; Hajek, M.; Frei, E.; Schmeiser, H. H. Carcinogenic and nephrotoxic alkaloids aristolochic acids upon activation by NADPH: Cytochrome P450 reductase form adducts found in DNA of patients with Chinese herbs nephropathy. *Gen. Physiol. Biophys.* **2001**, *20*, 375−392.
(29) Sun, C.; Sun, H.; Lai, Y.; Zhang, J.; Cai, Z. Liquid chromatography/mass spectrometry method for determination of perfluorooctane sulfonyl fluoride upon derivatization with benzylamine. *Anal. Chem.* **2011**, *83*, 5822−5826.
(30) Ye, Y.; Liu, H.; Horvatovich, P.; Chan, W. Liquid chromatography−electrospray ionization tandem mass spectrometric analysis of 2-alkylcyclobutanones in irradiated chicken by precolumn derivatization with hydroxylamine. *J. Agric. Food Chem.* **2013**, *61*, 5758−5763.
(31) Liu, J.; Chan, W. Quantification of Thiazolidine-4-carboxylic Acid in Toxicant-Exposed Cells by Isotope-Dilution Liquid Chromatography−Mass Spectrometry Reveals an Intrinsic Antagonistic Response to Oxidative Stress-Induced Toxicity. *Chem. Res. Toxicol.* **2015**, *28*, 394−400.
(32) Leung, E. M.; Chan, W. A novel reversed-phase HPLC method for the determination of urinary creatinine by precolumn derivatization with ethyl chloroformate: Comparative studies with the standard Jaffe and isotope-dilution mass spectrometric assays. *Anal. Bioanal. Chem.* **2014**, *406*, 1807−1812.
(33) Li, J.; Leung, E. M.; Choi, M. M.; Chan, W. Combination of pentafluorophenylhydrazine derivatization and isotope dilution LC−MS/MS techniques for the quantification of apurinic/apyrimidinic sites in cellular DNA. *Anal. Bioanal. Chem.* **2013**, *405*, 4059−4066.
(34) Wang, Y.; Chan, W. Determination of aristolochic acids by high-performance liquid chromatography with fluorescence detection. *J. Agric. Food Chem.* **2014**, *62*, 5859−5864.
(35) Hranjec, T.; Kovac, A.; Kos, J.; Mao, W.; Chen, J. J.; Grollman, A. P.; Jelakovic, B. Endemic nephropathy: The case for chronic poisoning by *Aristolochia*. *Croat. Med. J.* **2005**, *46*, 116−125.
(36) Ivanciuc, T.; Ivanciuc, O.; Klein, D. J. Modeling the bioconcentration factors and bioaccumulation factors of polychlorinated biphenyls with posetic quantitative super-structure/activity relationships (QSSAR). *Mol. Diversity* **2006**, *10*, 133−145.
(37) Liu, W.; Zhou, Q.; Zhang, Z.; Hua, T.; Cai, Z. Evaluation of cadmium phytoremediation potential in Chinese cabbage cultivars. *J. Agric. Food Chem.* **2011**, *59*, 8324−8330.

DOI: 10.1021/acs.jafc.6b02203
J. Agric. Food Chem. 2016, 64, 5928−5934