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Liquid chromatography–tandem mass spectrometry analysis of aristolochic acids in soil samples collected from Serbia: Link to Balkan endemic nephropathy

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Rationale: Over the past six decades, residents of farming villages in multiple countries of the Balkan peninsula have been suffering from a unique type of chronic renal disease, Balkan endemic nephropathy (BEN). It was speculated that environmental pollution by aristolochic acids (AAs) produced naturally by *Aristolochia clematitis* L., a weed that grows in the area, was causing the disease. However, the human exposure pathway to this class of phytotoxin remains obscure. Knowledge of the sink and stability of AAs in the environment would assist in the formulation of policy reducing exposure risk.

Methods: Using our newly developed liquid chromatography/tandem mass spectrometry method of high sensitivity and selectivity, we analysed over 130 soil samples collected from cultivation fields in southern Serbia for the presence of AAs. The environmental stability of AAs was also investigated by incubating soil samples spiked with AAs at various temperatures.

Results: The analysis detected AA-I in over two-fifths of the tested samples at sub-μg/kg to μg/kg levels, with higher concentrations observed in more acidic farmland soil. Furthermore, analysis of soil samples incubated at various temperatures revealed half-lives of over 2 months, indicating that AAs are relatively resistant to degradation.

Conclusions: Cultivation soil in southern Serbia is being extensively contaminated with AAs released from the decomposition of *A. clematitis* weeds. Since AAs are resistant to degradation, it is possible that AAs could have been taken up by root absorption and transported to the edible part of food crops. Prolonged exposure to AA-contaminated food grown from polluted soil could be one of the main aetiological mechanisms of BEN observed in the area.

1 | INTRODUCTION

Aristolochic acids (AAs; Figure 1) are a family of nitrophenanthrene carboxylic acids produced naturally in *Aristolochia* and *Asarum* plants,[1-3] which have long been widely used as herbal medicines for treating many inflammation-related diseases.[4-6] There is a significant amount of evidence suggesting that the prolonged intake of AAs through AA-containing herbal medicines is one of the major toxicological and carcinogenic triggers leading to the development of end-stage kidney disease and the upper tract urothelial cancers observed in patients suffering from aristolochic acid nephropathy (AAN),[7-9] an acute renal interstitial fibrosing disease observed all over the world.[10-12] AAN is predominantly caused by the inadvertent use of AA-containing herbs in preparing herbal remedies.[7-9]

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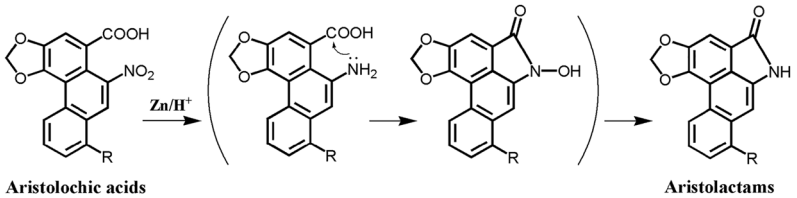

FIGURE 1 Zinc/H^+-induced nitroreduction converts AAs (AA-I, R = OCH_3; AA-II, R = H) into ALs of enhanced electrospray ionization MS response for LC/MS/MS analysis

Since the 1990s, the use of AA-containing herbs has been prohibited for preparing herbal medicines in many countries,[10] including the USA.[13] Despite the worldwide ban on the use of AA-containing *Aristolochia* plants as traditional herbal medicine and in herbal remedies,[10,12] it was estimated that over a hundred million people are potential victims of AA poisoning,[14] due to the traditional practice of Chinese herbal medicine,[2] the misuse of AA-containing herbs[7-9] and environmental exposure.[2,15-18]

Coincidentally, it is known that, for over 60 years, residents of rural farming villages alongside the Danube River across multiple countries of the Balkan peninsula have been suffering from a unique type of slowly progressive end-stage renal disease with clinical and morphological features similar to those of AAN.[2,19] The disease became known as Balkan endemic nephropathy (BEN), but its aetiology and pathogenic mechanism have been poorly understood until lately.[15-17,20] It has been estimated that over 25 000 residents of rural farming villages in Balkan areas are suffering from BEN and over 100 000 residents in these regions could be at risk.[14,16]

It was not until 2007 that AAs were identified to be the disease-causing agent of BEN.[21] It is suspected that BEN is an environmentally induced disease caused by the dietary intake of AA-tainted food.[15-17] Such food contamination was speculated to have been caused by the commingling of AA-containing seeds of *Aristolochia clematitis* L. (commonly known as birthwort) with wheat grains during the harvesting process. *A. clematitis* is a weed that grows abundantly among wheat in cultivated fields in the Balkan endemic areas.[2,16,22] However, the hypothesis was not supported by scientific evidence and the molecular mechanism by which AAs entered the human body remained obscure.[2]

There is some emerging evidence suggesting that AAs are environmental pollutants released into cultivation soil as the dead mass of *A. clematitis* decomposes.[15-18,23] Free AAs in the contaminated soil are then taken up by food crops via root absorption and translocated into their edible parts.[2,15,17] For example, AAs have recently been detected in cultivation soil and in food grains collected from wheat and maize collected from Serbian farmlands where *Aristolochia* weeds are present.[2,15-17] The contamination of food crops by AAs absorbed from polluted soil could be one of the major pathways by which AAs are transferred to humans.[15-17]

The goal of the study reported here was to obtain information on the geographical spread of AA contamination across a wider area and to understand the occurrence and environmental stability of AAs by quantifying them in soil samples collected from Serbia, one of the countries suffering from a high prevalence of BEN. Using a previously developed high-performance liquid chromatography coupled with tandem mass spectrometyr (LC/MS/MS) method,[23] we conducted the first large-scale surveillance of AAs in soil samples collected from farming villages in southern Serbia. These data provided direct evidence that soil in the cultivated fields of the Balkans is extensively contaminated with AAs. These results revealed an imperative need to control *A. clematitis* weeds in crop fields and for soil remediation for polluted farmlands.

2 | EXPERIMENTAL

2.1 | Caution

AAs are carcinogenic to humans and should be handled with care.

2.2 | Materials

AA, a mixture of aristolochic acid I (AA-I) and aristolochic acid II (AA-II) (1:1), was purchased from Acros Organics (Fair Lawn, NJ, USA). Benz[*cd*]indol-2(1*H*)-one, glacial acetic acid and zinc dust were obtained from Sigma Chemical Co. (St Louis, MO, USA). HPLC-grade methanol and LC–MS-grade acetonitrile were procured from Duksan (Gyeonggi-do, South Korea).

2.3 | Preparation of standard solutions

A primary stock solution of AA-I and AA-II in methanol was prepared at a concentration of 500 μg/mL for each AA and stored at −20°C. A secondary stock solution (1 μg/mL) was prepared by diluting the primary stock solution using water/methanol (1:4, v/v). Working standards at 4 to 60 ng/mL were prepared by adding an appropriate volume of the secondary stock solution to 10 mL of water/methanol (1:4, v/v) and stored at −20°C until required.

2.4 | Sample collection

Surface soil samples (n = 137; 5–10 cm) were collected manually from cultivation lands in farming villages in the vicinity of Niš, southern Serbia, in autumn 2018. Soil samples were taken from five villages in Serbia (Kutleš, Trupale, Brzi Brod, Medoševac and Popovac). After collection, all the samples were air-dried and stored at −20°C before further analysis.

2.5 | Sample preparation

Soil samples were processed as described previously,[17] with modifications. In brief, *ca* 0.4 g of sieved soil sample was accurately weighed and added with 4 mL of extraction solvent (methanol/water/acetic acid, 70/25/5 v/v) into a 15-mL screw-cap tube. The mixture was then subjected to ultrasonication at 50°C for 1 h to extract AAs from the soil. Following centrifugation at 6000 *g* for 10 min, 1 mL of the sample extract was reacted with 20 mg of pre-activated zinc dust at 60°C for 15 min to reduce AAs to their respective aristolactams (ALs; Figure 1).[15,17,23] After cooling to room temperature, 50 µL of internal standard, benz[*cd*]indol-2(1*H*)-one (200 ng/mL), was spiked to the samples, which were vortex-mixed and centrifuged at 13 800 *g* for 5 min prior to analysis using the LC/MS/MS method described below.

2.6 | Fortifications and calibration standards

Soil samples that were detected to have no AAs were pooled and used as sample blanks to prepare calibration standards. The AA-fortified samples were prepared at 1, 2.5, 5, 7.5, 10 and 15 µg/kg by adding 100 µL of the working standard of AAs to 0.4 g of blank soil ($n = 5$). The samples were vortex-mixed and air-dried in a hood for 6 h before subsequent treatment as described above. Calibration curves for quantitative analysis were constructed by plotting the peak area ratio of ALs to internal standard versus the concentration of AAs in the soil samples.

2.7 | Environmental stability of AAs

To investigate the stability of AAs in the environment, blank soil samples that were detected to have no AAs were first fortified with AAs and air-dried to obtain a final concentration of 100 µg/kg. The treated soil was then heated at 120°C overnight for sterilization purposes. Immediately, 6.0 g of the AA-spiked soil was added to beakers with 54.0 g of unsterilized soil and sterilized soil (heated at 120°C overnight before use) separately; the sterilized set was heated for a further 6 h to avoid any growth of bacteria. Two sets of soil were mixed thoroughly with a spatula and were incubated at room temperature (25°C, $n = 3$), 4°C ($n = 3$) and −20°C ($n = 3$) to investigate the stability of AAs in these conditions. On the day when the incubation was started and on 7, 14, 28, 42 and 60 days post-incubation, *ca* 0.4 g of the soil was sampled and analysed for the concentrations of AA-I and AA-II.

2.8 | LC/MS/MS analysis

The analyses were conducted using a Nexera X2 liquid chromatograph (Shimadzu, Kyoto, Japan) coupled to an API 4000 QTRAP tandem mass spectrometer (Sciex, Foster City, CA, USA). A VisionHT C18 HL column (100 mm × 2.1 mm, 3 µm; Grace, Deerfield, IL, USA) was used for the LC separation. An amount of 10 µL of the sample extract was injected into the column which was eluted with a binary solvent system of (A) 0.2% (v/v) acetic acid in water and (B) acetonitrile at 0.3 mL/min. The gradient was 0–10 min, 10–100% (v/v) B linear; 10–14 min, 100% B, followed by 4 min of re-equilibration of the column.

The mass spectrometer was operated in multiple reaction monitoring (MRM) mode using optimized positive electrospray ionization parameters.[23] The MRM transitions were set as follows: aristolactam I (AL-I, nitroreduction product of AA-I): m/z 294 → 279 and 294 → 251; aristolactam II (AL-II, nitroreduction product of AA-II): m/z 264 → 179 and 264 → 206; and internal standard benz[*cd*]indol-2(1*H*)-one: m/z 170 → 127. The dwell time for each transition was set at 100 ms.

2.9 | Method validation

The intra-day and inter-day reproducibility of the method were evaluated by analysing soil samples that were spiked with AA-I at 2, 6 and 10 µg/kg and AA-II at 6, 9 and 12.5 µg/kg, on the same day ($n = 7$) and over seven different days of a week ($n = 7$), respectively. The method accuracy was determined by dividing the measured by the spiked AA concentrations ($n = 3$). The method detection limits were calculated as the concentration of AA-I and AA-II generating a signal three times the noise level.[24]

2.10 | Measurement of soil pH

Soil pH analyses were performed according to the international standard procedure ISO 10390:2005.[17] In brief, soil samples were mixed with distilled water at a ratio of 1:5 (w/v) and agitated continuously for 1 h. After standing for 30 min, the pH of the soil/water mixture was measured with a calibrated pH meter (model 420A, Orion Research Inc., Boston, MA, USA).

3 | RESULTS AND DISCUSSION

3.1 | LC/MS/MS for quantification of AA-I and AA-II

We previously demonstrated that a combination of Zn/H^+-induced nitroreduction, solid-phase extraction (SPE) and LC coupled with fluorescence or tandem mass spectrometry analyses significantly enhanced the sensitivity of analysing AAs in environmental and food samples.[15,17,23] In particular, the analysis of AAs, in the form of nitroreduction derivatives ALs, by LC/MS/MS offers the highest sensitivity for the analysis of AAs in environmental and food samples.[23] Herein, an improved method is described that was developed and used for the surveillance of AAs in cultivation soil samples collected from Serbia. The simplified sample preparation method is described in detail in section 2, where the increased sensitivity and selectivity of the LC/MS/MS analysis allowed direct analysis of the sample extracts, without the SPE clean-up and enrichment step.

TABLE 1 Calibration parameters and minimum detection limits (MDLs) of the developed LC/MS/MS method for AA analysis

	AA-I	AA-II
Linear range (μg/kg)	1–15	5–15
Slope	0.06	0.01
Intercept	0.05	0.01
R^2	0.999	0.992
MDL (fg/injection)	250	1000
MDL (ng/kg)[a]	62.5	250

[a]MDL is the sample extract based on 400 mg of soil sample.

The modified sample preparation method combined with the newly developed LC/MS/MS method was applied to quantitate AAs in soil samples collected from cultivation fields in Serbia, where over 25 000 people living in farming villages along tributaries of the Danube River of the Balkan peninsula are suffering from BEN.[14] Despite its first discovery over 60 years ago, the true cause of BEN had remained hypothetical until recently, when scientists revealed that AAs are the main aetiological agents.[21] However, the exposure pathways by which humans are exposed to these nephrotoxins were not illuminated thoroughly.[15]

The goal of this study was to evaluate the geographical distribution of AA contamination across a wider region and thus contribute to correlating the role of AA pollution in the soil environment to the onset and development of kidney diseases among Balkan residents by analysing AAs in soil samples collected from several villages in Serbia. To this end, we first prepared standards by spiking soil samples with different amounts of AAs and analysed the samples using the developed method. The calibration parameters are presented in Table 1, indicating that both the extraction method and the Zn/H^+ treatment are qualitative for the captioned analysis. It is worth mentioning that we have demonstrated in a previous study that the yield of the Zn/H^+-induced reduction of AA-I and AA-II to produce their corresponding ALs is approaching 100%.[15] Furthermore, the higher selectivity of the MS/MS analysis allowed the detection of AAs in soil samples without the tedious and time-consuming SPE enrichment and clean-up step.

We then evaluated the accuracy and precision of the method by replicate analyses ($n = 7$) of soil samples spiked with various concentrations of AAs (Table 2). The results demonstrated that the method offered good accuracy (recovery: 94.6–102.3%) and precision (relative standard deviation: 1.2–10.7%; Table 2) for analysing AAs in soil samples. The detection limits of the method, estimated as the minimum concentration of AA-I and AA-II that would generate a signal three times the noise level,[24] were 62.5 and 250 ng/kg, respectively (Table 1).

3.2 | Quantitation of AA-I and AA-II in soil samples collected from Serbia

The validated method was then applied to quantitate AAs in cultivation soil samples ($n = 137$) collected from Serbia. Typical chromatograms from analysing AAs in soil samples are depicted in Figure 2. The analysis detected AA-I in 59 out of the 137 samples. After correcting to the concentration of native ALs (Table 3), which was determined by direct LC/MS/MS analysis of the sample extracts (without nitroreduction), it was found that AAs persist in the soil samples at concentrations ranging from 0.08 to 11.02 μg/kg. The results indicated that soil contamination by AAs released from decaying *A. clematitis* weed is a widespread and serious environmental problem in the area. The results from the analysis of 137 soil samples collected from farmlands in five different villages are summarized in Table 3.

It is worth mentioning that AA-II was detected at a lower frequency (in 1 out of the 137 samples; Table 3) and at a lower concentration (0.95 μg/kg). This observed difference could be attributed to the significantly lower concentration of AA-II than that of AA-I in the *A. clematitis* weed,[25] which also provided added evidence that the free AAs in the soil are released from nearby decaying *A. clematitis*. With the previous studies showing that the free AAs in the soil can be absorbed and accumulated in food crops,[15,17] which are the staple food for local residents, it is imperative to research for environmental stability of AAs and methods for remediation.

Furthermore, the average concentration of AA-I detected in the present study (1.26 μg/kg) was lower than that reported previously (52.60 μg/kg).[17] This is attributed to the newly developed method with higher sensitivity allowing the detection of AAs at significantly lower concentrations, and in many of the samples AAs were detected at sub-μg/kg concentrations. For the same reason, AA is detected in a slightly higher frequency (43%) than that reported

TABLE 2 Validation of LC/MS/MS method for soil sample analysis

Concentration added (μg/kg)		Precision		Accuracy	
		Intra-day (% RSD)[a]	Inter-day (% RSD)[a]	Concentration found (μg/kg)[b]	Recovery (%)
AA-I	2	3.8	6.5	1.89 ± 0.18	94.7 ± 9.0
	6	4.6	8.7	5.75 ± 0.13	95.7 ± 2.1
	10	2.7	3.2	10.23 ± 0.29	102.3 ± 2.9
AA-II	6	7.3	10.7	5.68 ± 0.20	94.6 ± 3.3
	9	3.6	4.6	8.83 ± 0.10	98.2 ± 1.1
	12.5	1.2	3.9	12.43 ± 0.25	99.4 ± 2.0

[a]$n = 7$.

[b]$n = 3$.

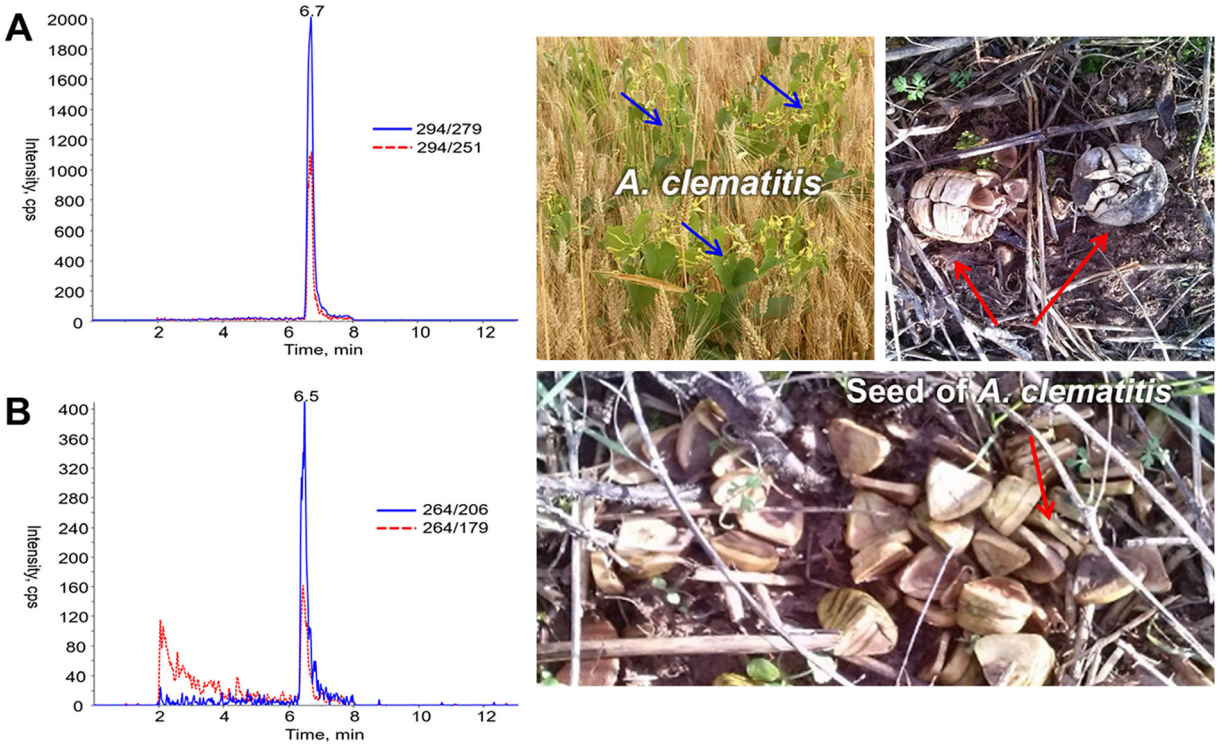

FIGURE 2 Typical chromatograms obtained from LC/MS/MS analysis of A, AA-I and B, AA-II in a soil sample containing 2.7 µg/kg of AA-I and 1.0 µg/kg of AA-II. C, Photos (taken in summer 2016) showing *A. clematitis* weeds growing in a wheat field in a typical village (Kutleš) in Serbia, together with photos (taken in autumn 2016) showing decaying seeds of *A. clematitis* in the field [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Concentrations of AAs in soil samples collected in Serbia

	AA-I	AA-II	AL-I	AL-II
No. of samples analysed	137			
No. of positive samples[a]	59	1	32	0
Concentration range (µg/kg)	0.08–11.02	0.95	0.22–5.07	–
Average concentration (µg/kg)	1.26 ± 1.73	–	1.41 ± 1.15	–

[a]Samples with concentrations above the method detection limit: 62.5 ng/kg of AA-I, 250 ng/kg of AA-II.

previously (36%).[17] Nevertheless, the results showed soil contamination by AAs released from decaying *A. clematitis* is an alarming problem in the area. With the prior study showing that AAs can be taken up by root absorption of food crops and translocated into edible parts,[15,17] it is reasonable to deduce that the frequent consumption of staple food in the region made with grains contaminated with AAs is one of the leading causes of the many BEN kidney disease cases observed.

3.3 | Correlation between concentration of AA-I and soil pH

Having determined the concentrations of AAs in the soil, we assessed the correlation to soil acidity. To this end, we measured the soil pH using the ISO 10390:2005 method.[17] The pH values of those soil samples with positive identification of AAs ($n = 59$) ranged from 5.0 to 8.3. Figure 3 shows the values of AA-I availability as a function of soil pH. The results showed that the concentration of AA-I in soil decreased gradually from the most acidic to the most alkaline pH values. The higher level of AA-I in the acidic environment could be attributed to the higher density of *Aristolochia* plants growing in the surrounding environment, since the germination of *Aristolochia* plants is known to be favoured in a slightly acidic environment.[17,26] The data are also in good agreement with previous results that AAs were detected at higher concentrations in farmland soil with lower pH values.[17] These observations may lead to the development of new methods to reduce or eliminate AAs from farmland soil through soil pH adjustments, such as to increase the soil pH by adding alkaline gardening lime (calcium hydroxide).

3.4 | Stability of AA-I and AA-II in the environment

Having determined the concentration of AAs in the soil samples collected from rural farming villages in Serbia, we then investigated

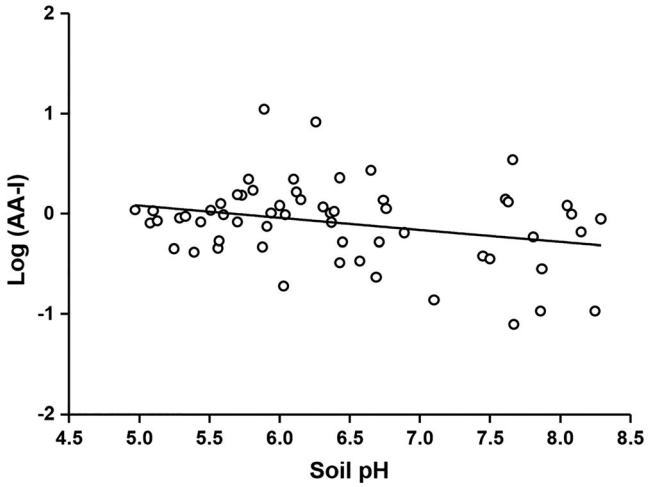

FIGURE 3 Logarithm of soil concentration of AA-I as a function of soil pH in soil samples collected from farmlands in Serbian villages ($n = 59$)

the environmental stability of AAs in the agricultural soil. To mimic the weather conditions of the Balkan peninsula across the year, we incubated soil samples collected from cultivation fields in Serbia that were spiked with 10 µg/kg of AA-I and AA-II at 25, 4 and −20°C. The soil samples were sampled at different time points to investigate the effect of soil microbial activity on AAs.

Our study showed that the concentrations of both AA-I and AA-II decreased at a faster rate at room temperature than in the samples stored at 4 and −20°C (Figure 4). During the 60 days of incubation, the concentration of AAs dropped by *ca* 50% at room temperature, whereas the AA levels were comparatively more stable at 4 and −20°C with more than *ca* 60% of AAs remaining. The temperature dependency observed here suggests that the persistence of AAs in soil could be related to microbial activities,[27,28] since microbes are much less active at colder temperatures.[29,30] It was also observed that slower degradation rates of AAs were detected for the heat-treated, microbial-free samples at all temperatures: *ca* 10–20%

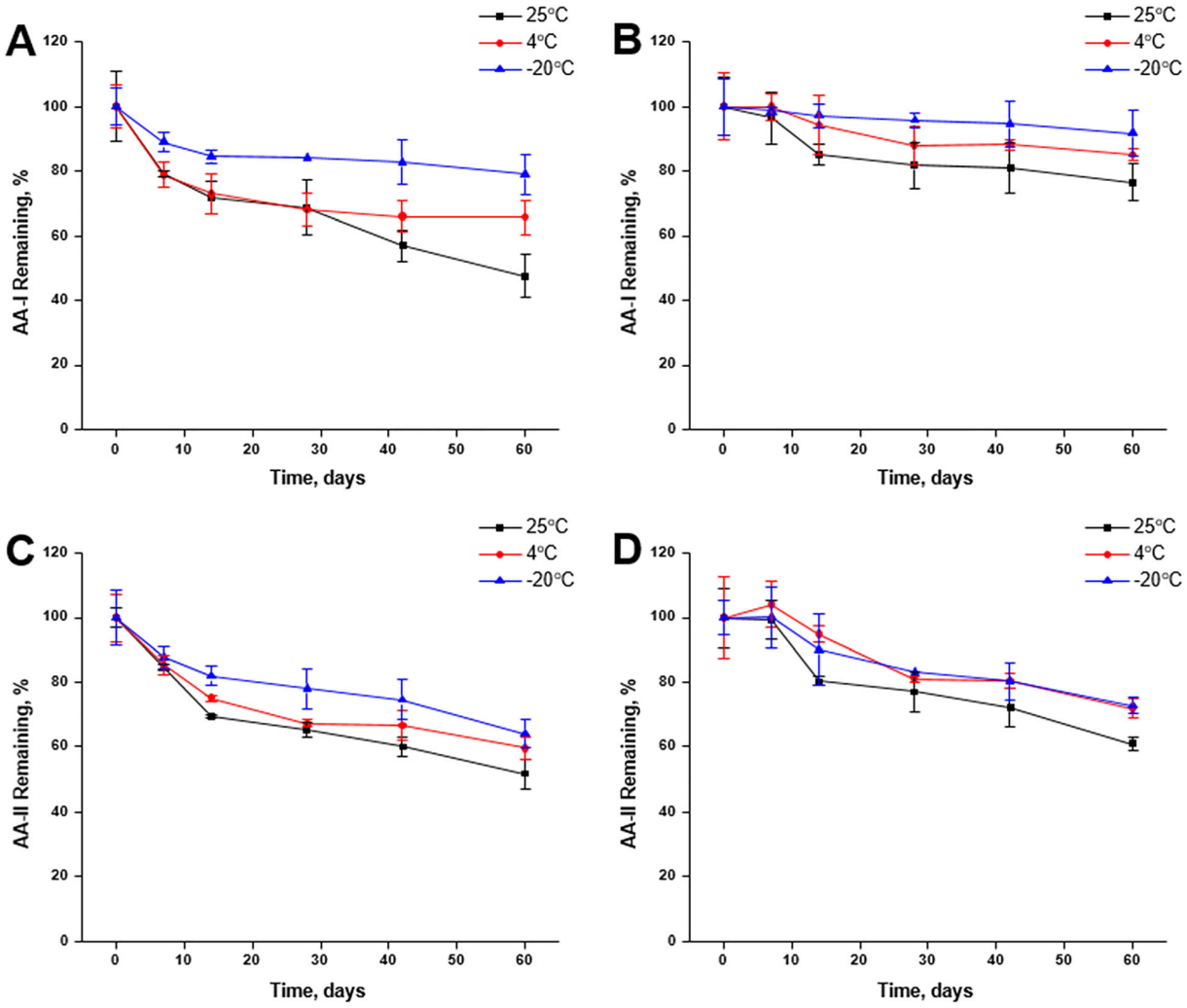

FIGURE 4 Environmental stability of A, AA-I and B, AA-II in soil. Blank soil samples collected from Serbia were spiked with a mixture of AA-I and AA-II at 10 µg/kg. The soil samples were analysed by LC/MS/MS after 7, 14, 28, 42 and 60 days of storage at 25, 4 or −20°C. The concentration at day 0 was taken as the control. Concentrations of C, AA-I and D, AA-II in heat-treated soil samples [Color figure can be viewed at wileyonlinelibrary.com]

more AAs remained than in the original soil without heat treatment (Figure 4). These results further confirm that the degradation of soil AAs is likely to be mediated through soil microbes and their activities. It is also reasonable to speculate that remediation could be carried out with selected microbes. A similar bioremediation process was also observed for polycyclic aromatic hydrocarbon compounds and heavy metals.[31-35] Our results also agree with those of a previous study that AA-II is less stable than AA-I in soil, based on the lower levels of AA-II remaining in all the experimental soil sets.[17]

Despite AAs being degraded at a reasonable rate at 25°C by yet-to-be-determined microorganisms and to some unknown metabolites, winter in Serbia and most countries in the Balkan peninsula is long and cold where AAs could be degraded relatively slowly. It is possible that AAs will persist in the environment and get taken up by root absorption. Therefore, it is imperative to eliminate *A. clematitis* weeds from cultivation fields and to research for remediation methods.

4 | CONCLUSIONS

Using our newly developed LC/MS/MS method, we performed a large-scale surveillance of AAs in cultivation soils collected from multiple farming villages in southern Serbia. The analyses detected AA-I at low μg/kg levels in 59 of the 137 soil samples tested, whereas AA-II was detected at slightly lower concentration and occurrence frequency. The results from this study revealed that cultivation soil in Serbia is extensively contaminated by AAs released from the decomposition of the AA-containing *A. clematitis* weed that grows widely in the area. Residents in the affected areas are continuously exposed to this class of nephrotoxin and carcinogen. It is highly possible that chronic exposure to AAs through contaminated food is one of the major causes of BEN. It is imperative to eliminate *A. clematitis* weed from cultivation fields and to research for efficient remediation methods.

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