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Development of a novel liquid chromatography-tandem mass spectrometric method for aristolochic acids detection: Application in food and agricultural soil analyses

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ABSTRACT

Prolonged dietary exposure to AA-contaminated food crops cultivated in contaminated farmland was shown to be one of the main culprits of Balkan endemic nephropathy. Ultra-sensitive methods for the detection of AA at trace levels are important for surveillance purposes. We report the development of a novel liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for AA-detection. One striking feature of the method is that the Zn/H^+-induced nitroreduction convert AA into their respective aristolactams, which dramatically enhances their analytical sensitivity. The method was applied to quantitate AA in food grains and soil samples collected from farming villages of Serbia, and flour samples purchased from supermarkets. To the best of our knowledge, this is the first report of detecting AA in food ingredients available in supermarkets, which is alarming because it reveals AA poses insidious threats not only to residents of rural farming villages, but also to people residing in urban areas.

1. Introduction

Aristolochic acids (AA) are a family of structurally related nitrophenanthrene carboxylic acids naturally produced by *Aristolochia* and *Asarum* plants, with AA-I and AA-II being the predominant members (Fig. 1) (Stiborová, Arlt, & Schmeiser, 2016). Since ancient times, AA-containing herbs have been widely used as herbal medicines and ingredients to prepare proprietary Chinese medicine and dietary supplements (Grollman & Marcus, 2016); however, in the late 1980s, AA were discovered to be highly nephrotoxic and carcinogenic in rodent models (Mengs, 1987, 1988). Since then, the US Food and Drug Administration has urged the manufacturers and distributors of botanical products to ensure that their products are free from AA (Poon et al., 2015). It was also suggested that consumers immediately stop using botanical products that contain or might contain AA. Currently, AA and plants containing them have been classified by the International Agency for Research on Cancer as Group 1 carcinogens (Arlt, Stiborová, & Schmeiser, 2002; Debelle, Vanherweghem, & Nortier, 2008), and the sales of AA-containing herbal remedies are prohibited in multiple countries, including Australia, Canada, Germany, the UK, the USA, and New Zealand (Martena et al., 2007).

Unfortunately, the misuse of AA-containing herbs in the preparation of health products and herbal medicines has been sporadically reported worldwide (Chen et al., 2012), with the most alarming incident occurring in Belgium in the 1990s. A further investigation revealed that an AA-containing herb, *Aristolochia fangchi*, was inadvertently used to prepare slimming pills and caused ~100 women who participated in the regimen to suffer from a unique type of kidney fibrosis; over half of these women underwent renal replacement therapy (Arlt et al., 2002). This unique type of kidney disease with rapid progression was originally named Chinese herb nephropathy and later renamed aristolochic acid nephropathy (AAN) for a more accurate representation of its true etiology (Cosyns, 2003).

For over 60 years, tens of thousands of residents of rural farming villages in multiple countries of the Balkan Peninsula have suffered from a unique type of chronic progressive renal disease known as Balkan endemic nephropathy (BEN), which has clinical and morphological parameters similar to those observed in AAN patients (Cosyns, 1994, 2003; Grollman, 2013). Despite tremendous efforts to investigate the origin of the disease, its cause is still mysterious. Recent work by Grollman et al. has shed light on this topic in which DNA-AA adducts in DNA samples isolated from the kidney tissue of BEN patients were identified, thus establishing a molecular link between AA and BEN development (Grollman et al., 2007). It was also discovered that food

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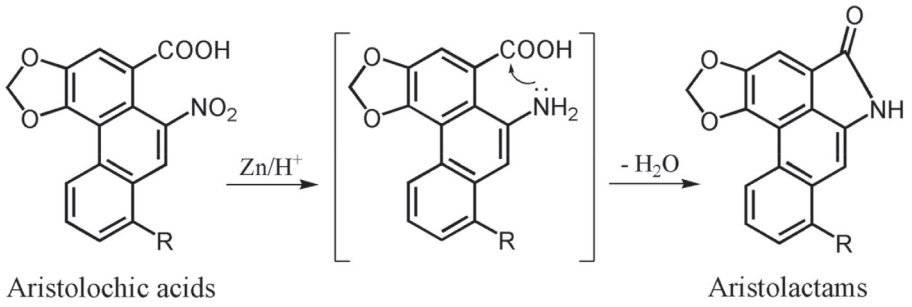

Fig. 1. Zinc/H^+-induced nitroreduction of converts aristolochic acids to aristolactams of enhanced ESI-MS response for LC–MS/MS analysis. AA-I, R = OCH_3; AA-II, R = H.

and environmental pollution by AA released from decaying *Aristolochia clematitis* L., an AA-containing weed growing extensively in cultivated fields in the Balkan Peninsula, is the culprit (Chan et al., 2016; Gruia et al., 2017; Li et al., 2018). Accumulating evidence shows that prolonged intake of AA-contaminated food products made from food grains grown in the polluted area is one of the main causes of the disease (Chan et al., 2016; Li et al., 2018; Pavlović et al., 2013). Therefore, sensitive methods for AA detection are necessary to detect AA as novel contaminants in food and environmental samples.

Current approaches for AA detection include capillary electrophoresis coupled with ultraviolet absorption or electrochemical detection and high-performance liquid chromatography (HPLC) coupled with ultraviolet or fluorescence detection (Chan, Lee, Liu, & Cai, 2007; Li et al., 2018; Wang & Chan, 2014). Most of these methods are used to quantitate AA in herbal remedies, which contain relatively high concentrations of AA, but they may not be sufficiently sensitive to detect low levels (μg/kg) of AA in the complex matrices of food and environmental samples. In addition, the analysis of AA in a complex matrix by the existing methods requires further validation experiments to achieve a reliable compound identification (Chan et al., 2016; Li, Hu, & Chan, 2016).

To meet the emerging challenge of detecting AA in the complex matrix of food products and agricultural soil samples, there is a need for enhanced analytical sensitivity and specificity for the analysis. Mass spectrometry is one of the most sensitive and selective analytical methods, and it is commonly used in food contaminant and environmental analyses (Hollender, Schymanski, Singer, & Ferguson, 2017). However, the application of mass spectrometry in AA analysis is limited (Kite, Yule, Leon, & Simmonds, 2002; Kuo et al., 2010; Sorenson & Sullivan, 2007; Yu et al., 2016), probably due to the low ionization efficiency, which hampers the detection of AA when using electrospray ionization-mass spectrometry (ESI-MS) (Yuan et al., 2007, 2017).

To address these challenges, a novel LC–MS/MS method was developed with the lowest LOD and LOQ values when compared to other methods that are available in literature. Our goal is to develop a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method with higher sensitivity for the quantification of AA by lowering the method detection limit to allow trace or ultra-trace AA analysis in food grains and soil samples collected from contaminated areas. To do so, we used a simple approach that chemically reduces AA to their corresponding aristolactams (AL) with higher ionizability in positive ESI-MS (Fig. 1), which we have used in previous studies for converting the non-fluorescencing AA to fluorescencing AL for HPLC-fluorescence detection (Chan et al., 2007, 2016; Li et al., 2018).

2. Materials and methods

2.1. Caution

AA are nephrotoxic and carcinogenic to humans and should be handled with care.

2.2. Chemicals and reagents

All chemicals and reagents were of the highest purity available and were used without further purification. AA (mixture of AA-I and AA-II, 1:1) were obtained from Acros (Morris Plains, NJ). Benz[*cd*]indol-2(1*H*)-one, zinc dust, and glacial acetic acid were acquired from Sigma Chemical Co. (St. Louis, MO). Aristolactam standards (mixture of AL-I and AL-II, 1:1) were prepared by nitroreduction with Zn/H^+ at ambient temperature, as described previously (Li et al., 2016). HPLC-grade methanol and acetonitrile were purchased from Tedia (Fairfield, OH). Deionized water was further purified by a Milli-Q Ultrapure water purification system (Billerica, MA) and used in all experiments. C18 solid-phase extraction (SPE) columns packed with 500 mg of sorbents were obtained from Grace (Deerfield, IL).

2.3. Sample collection

Wheat grain (n = 20), maize grain (n = 20), and farmland soil (n = 20) samples were collected in Serbia (village Kutleš; latitude, 43.140; longitude, 21.862; and elevation, 211 m). Wheat (n = 36) and maize (n = 24) flour samples (total n = 60) were purchased from supermarkets in Serbia and Bulgaria. Control wheat and maize flour samples (n = 18) were obtained from supermarkets in Hong Kong.

2.4. Sample preparation

Using our previously developed sample preparation method (Chan et al., 2016; Li et al., 2016), food grains, flour and soil samples were processed for later analysis. Briefly, 2 mL of extraction solvent (methanol/water/acetic acid: 70/25/5, v/v) was added to 200 mg of homogenized samples and sonicated at 50 °C for 1 h. One milliliter of supernatant was then added to 20 mg of Zn dust and agitated with heating at 60 °C for 15 min to reduce AA to AL; finally, sample clean-up was performed on a C18 SPE cartridge (Chan et al., 2016; Li et al., 2016). To the purified sample solution, 10 μL of benz[*cd*]indol-2(1*H*)-one (0.12 μM) was added as an internal standard, followed by drying with nitrogen stream. The residual was eventually dissolved in 100 μL of methanol for LC-FLD or LC–MS/MS analysis. Using a similar procedure but without the Zn/H^+ treatment step, the concentrations of naturally occurring AL in the samples were also determined (Fig. S1).

2.5. Instrumental analysis

LC-FLD analysis was performed on a Thermo Ultimate 3000 HPLC system coupled to a programmable fluorescence detector (Waltham, MA), as described previously (Chan, Wong, & Li, 2018). The HPLC system consisted of a YMC Hydrosphere C18 column (150 mm × 3.0 mm, 3 μm) eluted at 0.35 mL/min with the following gradient of acetonitrile in water: 0–14 min, 35 to 100% (v/v); 14–19 min, 100% (v/v); and 19–25 min, 35% (v/v). The excitation and emission wavelengths of the FLD were set at 254 and 451 nm,

respectively. LC–MS/MS detection of AA and AL was performed on a Shimadzu Nexera X2 LC (Kyoto, Japan) coupled to an AB Sciex API 4000 QTRAP tandem mass spectrometer with a standard Turbo V Ion Source (Foster City, CA). A Grace VisionHT C18 HL column (100 × 2.1 mm, 3 µm; Deerfield, IL) was used for the chromatographic separation, which was eluted at a constant flow rate of 0.3 mL/min using a gradient elution program (10–100% acetonitrile in 9 min) with 0.2% acetic acid in water (for AL analysis) or 0.1% ammonium acetate in water (pH 7.4; for AA analysis) and acetonitrile as the mobile phase.

MS data were acquired in positive ESI mode using the optimized ionization source parameters. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. The MRM transitions for AA-I, AA-II, AL-I, AL-II and the internal standard were set as follows: AA-I: *m/z* 359 → 298 and 359 → 324; AA-II: *m/z* 329 → 268 and 329 → 294; AL-I: *m/z* 294 → 279 and 294 → 251; AL-II: *m/z* 264 → 179 and 264 → 206; and benz[*cd*]indol-2(1*H*)-one: *m/z* 170 → 127 (Fig. S2). The dwell time for each transition was set at 100 ms. The optimized voltage, source and gas parameters for AA and AL are listed in Table S1.

2.6. Method calibration and validation

The matrix-matched calibration method, which corrects the matrix effect on LC-FLD and LC–MS/MS analyses, was adopted. Control wheat and maize grain/flour samples that were tested and found to be free of AA were extracted to prepare blank sample extracts. Working standard solution mixtures of AL-I and AL-II at five concentrations ranging from 0.1 to 50 ng/mL and from 0.2 to 50 ng/mL (n = 3), respectively, were prepared by serial dilution of a stock solution mixture of AL-I (1 µg/mL) and AL-II (1 µg/mL) using blank sample extracts. The detection limits were estimated by successive dilution of the AL working standard using blank sample extracts until a signal-to-nose level of 3 was achieved in the LC–MS/MS analysis.

The method precision was evaluated by analyzing blank samples spiked with AA (0.1 mL) at three different concentrations (grain samples: 2, 10 and 50 ng/g; flour samples: 2.5, 25 and 75 ng/g) on the same day (n = 7) and over seven separate days in two weeks. The method accuracy was determined by analyzing AA-fortified samples at three different concentrations in blank grain and flour samples (n = 3, Table S2), and the amount of AA recovered were compared with that of the spiked levels to estimate the method recovery.

3. Results and discussion

3.1. LC–MS/MS characterization of the nitroreduction product of AA

LC–MS/MS is an inherently sensitive and selective method for food and environmental analysis (Hollender et al., 2017); however, its application for analyzing nephrotoxic and carcinogenic AA is limited (Kite et al., 2002; Kuo et al., 2010; Sorenson & Sullivan, 2007; Yu et al., 2016). Although AA possess carboxyl groups in their structures that deprotonate easily to generate negatively charged carboxylate ions for MS analysis, they produce poor signals when analyzed with negative ESI-MS (~1000 times lower than that in positive ESI-MS) (Kite et al., 2002). Unfortunately, in positive ESI-MS analysis, the low proton affinity of AA also lowered the ionization efficiency, which hinders the performance of AA analysis. To this end, direct LC–MS/MS analysis of AA usually monitors $[M+NH_4]^+$ ions instead of $[M+H]^+$ ions to achieve an eight-fold higher sensitivity than that of protonation (Kite et al., 2002), although the sensitivity is still lower than those previously reported with LC-FLD methods (Chan et al., 2016; Li et al., 2016; Wang & Chan, 2014). In addition, the carboxyl groups on AA are fragile and are fragmented easily in the ion sources of MS, even when soft ESI technology is applied, leading to even lower analytical sensitivity for AA in ESI-MS analysis.

To solve these problems, which are currently limited to LC-UV and LC-FLD analyses, we adopted a chemical reduction approach to transform AA to AL for enhanced ESI-MS responses (Fig. 1), which we have used in previous studies for converting the non-fluorescencing AA to fluorescencing AL for HPLC-fluorescence detection (Chan et al., 2007). Zinc dust in an acidic medium (Zn/H^+) first induces reduction of the nitro group in AA to an amino group, which then undergoes an intramolecular acetylation reaction with neighboring carboxyl groups to form stable lactams (Fig. 1) (Coutts, Stenlake, & Williams, 1957). Using this simple and efficient nitroreduction, non-fluorescing AA are converted to fluorescing AL, enabling enhanced detection by HPLC with a fluorescence detector (FLD) (Chan et al., 2007, 2016; Gillerot et al., 2001; Li et al., 2016; Yuan et al., 2008).

Here, we show for the first time that the formation of AL by nitroreduction of AA also facilitates their detection by LC-ESI-MS/MS. This approach provides single-digit femtomolar sensitivity for the identification of AA in food grain and soil samples collected from Serbia; an increase in the sensitivity of the method compared with other methods further allow us to detect, for the first time, AA in supermarket flour samples collected in Serbia, potentially indicating widespread food contamination by AA in the community. AL are stable reductive metabolites of AA that were detected *in vitro* and in urine samples of AA-exposed rats (Chan, Zheng, & Cai, 2007; Jadot, Declèves, Nortier, & Caron, 2017). The results showed that AA can be efficiently reduced to AL (> 99% yield) upon gentle heating with Zn/H^+. This condition is therefore adopted in this study to convert AA to their corresponding AL for LC–MS/MS analysis using ESI as the ionization technique.

Collision-induced dissociation of the $[M+H]^+$ ion of the AL-I at *m/z* 294 led to the formation of major fragment ions at *m/z* 279 resulting from the fragmentation loss of a methyl group (Fig. 2A); other fragment ions at *m/z* 251 were also observed and arose from the loss of the lactam ring. Collision-induced dissociation analysis of AL-II produced fragment ions at *m/z* 206 and 179 (Fig. 2B), which were consistent with those observed in previous studies. The fragment ions with the highest abundance (*m/z* 279 for AL-I; *m/z* 206 for AL-II) were selected for the

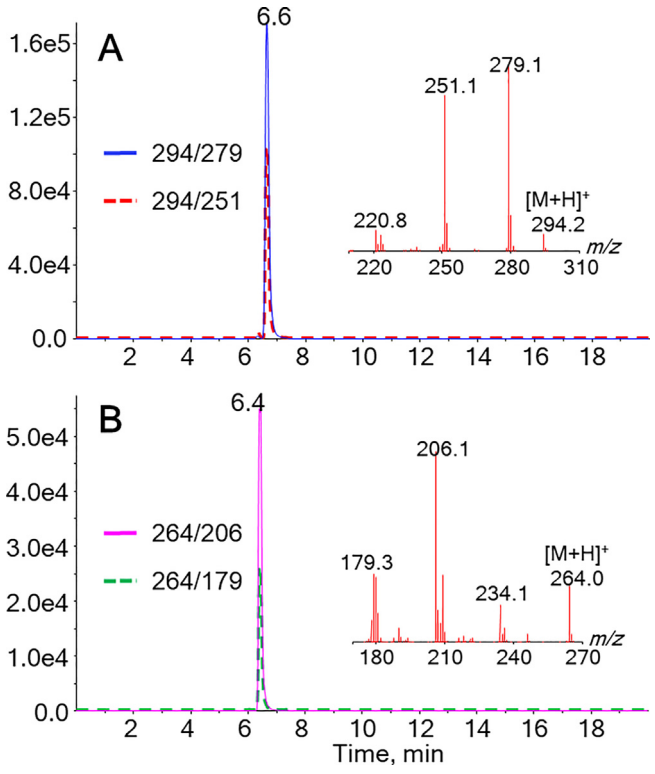

Fig. 2. Extracted ion chromatograms at MRM transitions of (A) *m/z* 294 → 274 and 294 → 251 for aristolactam I; and (B) *m/z* 264 → 206 and 264 → 179 for aristolactam II. Shown in the insets are the product ion spectra of the $[M+H]^+$ ion of aristolactam I (A, *m/z* 294) and aristolactam II (B, *m/z* 264).

Table 1
Comparison of the Limit of Detection of the Developed LC–MS/MS Method and other Methods Reported in the Literature for AA Detection.

	AA-I		AA-II		Reference
	LOD[c], ng/mL	LOD ratio	LOD, ng/mL	LOD ratio	
LC–MS/MS[a]	0.02	N/A	0.04	N/A	*Current*
LC-FLD	0.27	14	0.54	14	Wang and Chan (2014)
LC-FLD	0.39	20	0.52	14	Chan et al. (2007)
LC-FLD	0.73	37	0.92	24	Li et al. (2016)
LC–MS/MS[b]	12.0	600	10.0	263	Yuan et al. (2007)
LC–MS/MS[b]	12.0	600	15.0	395	Chan et al. (2003)
LC-UV	13.64	682	31.1	818	Ong, Woo, and Yong (2000)
CE-UV	320	16,000	100	2631	Ong and Woo (2001)
CE-ECD	1200	60,000	900	23,684	Zhou, Zheng, Sun, and You (2006)

[a] LC–MS/MS analysis of AL with pre-column derivatization with Zn/H^+.
[b] Direct LC–MS/MS analysis of AA without pre-column derivatization with Zn/H^+.
[c] Lower limit of detection estimated as the concentration of AA that generate a signal three times the noise level.

Table 2
Concentrations of AA-I and AA-II in Soil, Grain Samples Collected from Endemic Village Kutleš, Serbia (43° 8′ 22.93″ N, 21° 51′ 39.44″ E) and Flour Samples Collected from Supermarkets in Serbia Analyzed by using Different Methods.

	AA-I, (ng/g)[c]			AA-II, (ng/g)[c]		
	LC–MS/MS[a]	LC-FLD	LC–MS/MS[b]	LC–MS/MS[a]	LC-FLD	LC–MS/MS[b]
Wheat grain[d]	80.9 ± 11.2	96.2 ± 17.8	84.5 ± 9.3	79. 0 ± 12.1	81.6 ± 9.5	76.9 ± 13.7
	14.7 ± 2.4	17.2 ± 3.0	12.6 ± 2.0	10.2 ± 2.0	13.9 ± 1.6	9.33 ± 1.82
	2.18 ± 0.34	1.74 ± 0.27	NQ[f]	1.72 ± 0.19	1.57 ± 0.28	NQ
	0.69 ± 0.11	NQ	NQ	0.53 ± 0.08	NQ	ND
	0.13 ± 0.04	ND	ND[g]	NQ	ND	ND
Maize grain[d]	20.59 ± 3.7	21.2 ± 2.6	19.4 ± 3.2	18.0 ± 1.6	20.4 ± 2.1	18.8 ± 4.3
	3.26 ± 0.64	2.69 ± 0.49	2.89 ± 0.32	2.17 ± 0.33	2.24 ± 0.40	NQ
	0.43 ± 0.11	NQ	ND	0.33 ± 0.05	ND	ND
	0.31 ± 0.04	NQ	ND	0.22 ± 0.03	ND	ND
Agricultural soil[d]	23.60 ± 5.2	20.1 ± 2.9	20.4 ± 3.10	15.3 ± 2.2	14.1 ± 1.8	15.1 ± 1.9
	1.79 ± 0.29	1.64 ± 0.22	NQ	1.71 ± 0.33	1.77 ± 0.19	NQ
	0.23 ± 0.04	ND	ND	0.22 ± 0.03	ND	ND
	0.21 ± 0.02	ND	ND	NQ	ND	ND
Wheat flour[e]	0.56 ± 0.12	NQ	ND	ND	ND	ND
	0.39 ± 0.05	NQ	ND	ND	ND	ND
	0.37 ± 0.03	NQ	ND	ND	ND	ND
	0.23 ± 0.05	NQ	ND	ND	ND	ND
	0.23 ± 0.04	NQ	ND	ND	ND	ND
	0.16 ± 0.04	ND	ND	ND	ND	ND
	0.15 ± 0.03	ND	ND	ND	ND	ND
Maize flour[e]	0.48 ± 0.09	NQ	ND	ND	ND	ND

[a] LC–MS/MS analysis of AL with pre-column derivatization with Zn/H^+.
[b] Direct LC–MS/MS analysis of AA without pre-column derivatization with Zn/H^+.
[c] Processed and analyzed three times.
[d] Collected from farming village in Serbia.
[e] Collected from local supermarket in Serbia.
[f] Detected but cannot be quantified.
[g] Not detected. N.B. Results from samples with AA concentration lower than limit of quantitation/detection on LC–MS/MS[a] analysis were not shown here.

quantitative analysis of AA by MRM mode of a tandem mass spectrometry. The fragment ions at *m/z* 251 and 179 were used for the qualitative analysis of AA-I and AA-II, respectively. Under the chromatographic conditions described in the Experimental section, the nitroreduction products of AA-I and AA-II were eluted at retention times of 6.7 min (Fig. 2A) and 6.4 min, respectively (Fig. 2B).

3.2. Linearity, recovery, reproducibility, accuracy, and detection limits

The matrix-matched working standard solutions were analyzed using the LC–MS/MS method described above, and the peak area ratio of AL to that of the internal standard was used to establish the calibration curve. The LC–MS/MS method for AA-I and AA-II determination was found to be linear over the concentration range of 0.1–50 ng/mL and 0.2–50 ng/mL, respectively. Fitting the data by linear regression yielded lines with the equations $y = 1.42x - 0.38$ ($r^2 = 0.9998$) and $y = 0.46x - 0.15$ ($r^2 = 0.9996$) for AA-I and AA-II, respectively.

The analytical recovery was determined by spiking a mixture of AA into blank samples (grain samples: 2, 10 and 50 ng/g; flour samples: 2.5, 25 and 75 ng/g), followed by extraction, Zn/H^+ treatment, SPE cleanup, and LC–MSMS analysis, as described above. The recovery for both AA-I and AA-II were89.8 ± 9.9% ($n = 3$) and 89.3 ± 10.2% ($n = 3$), respectively, indicating that both the extraction and SPE processes are highly quantitative (Table S2).

The method precision was evaluated by analyzing blank samples spiked with AA at three different concentrations (grain samples: 2, 10 and 50 ng/g; flour samples: 2.5, 25 and 75 ng/g) on the same day ($n = 7$) and over seven separate days in two weeks. The intraday precision of the assay at the three spiked levels had a standard deviation of the peak area less than 8.5% for both AA-I and AA-II ($n = 7$, Table S2). Over a period of 2 weeks, the reproducibility of the developed method for the AA determination varied by less than 10.7% ($n = 7$). The

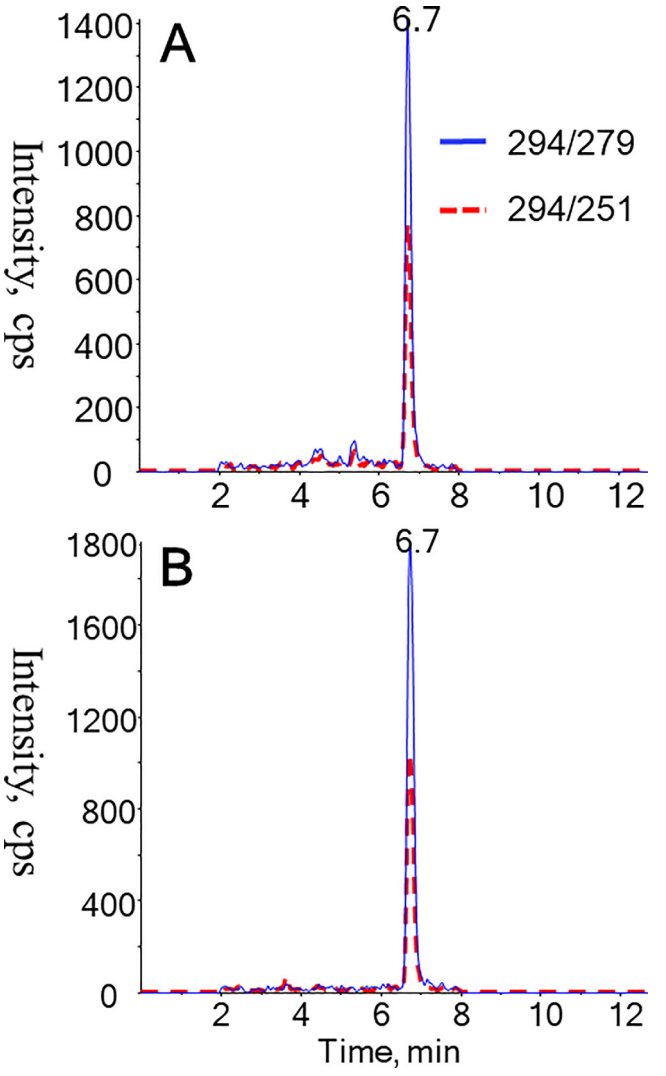

Fig. 3. Typical LC–MS/MS chromatograms from MRM of the nitroreduction derivative of AA-I (aristolactam I, *m/z* 294 → 274 and 294 → 251) in (A) wheat flour and (B) maize flour sample purchased from in supermarkets in Niš, Serbia. Aristolactam I was eluted at 6.7 min under the chromatographic condition described in the Materials and Methods section. Aristolactam II is with concentration below the method detection limit.

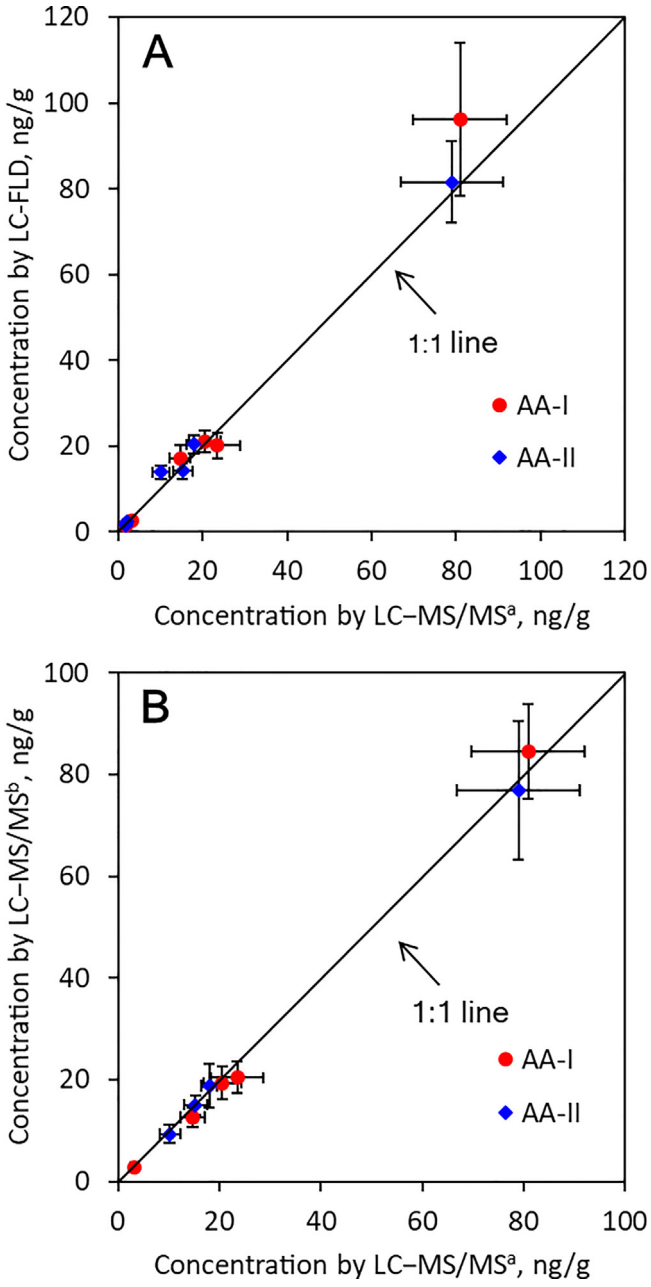

Fig. 4. Method validation by comparison of AA-I and AA-II concentrations in wheat grain collected from an endemic village in Serbia (n = 20) measured by (a) LC-FLD method against the newly developed LC–MS/MS method; and (B) by direct LC–MS/MS analysis against the newly developed LC–MS/MS method with nitroreduction. Fitting the data by linear regression yielded lines with the regression coefficient r^2 = 1.0.

method accuracy, as determined by analyzing spiked samples with three different concentrations of AA in blank samples (n = 7), was less than 10.2% and 10.7% for AA-I and AA-II, respectively (Table S2).

The detection limits for AA-I and AA-II, at a signal-to-noise ratio of 3, were determined as 0.02 ng/mL (0.04 ng/g grain or flour or soil) and 0.04 ng/mL (0.08 ng/g grain or flour or soil), respectively. These values are at least 14 and 260 times lower than those in the existing LC-FLD and LC–MS/MS methods, respectively, for the quantification of AA (Table 1).

3.3. Application in food grain and soil sample analysis

The validated LC–MS/MS method was then applied for the determination of AA in wheat grain (n = 20), maize grain (n = 20) and farmland soil (n = 20) samples collected from farming villages of Serbia (Table 2) where AA were previously detected (Chan et al., 2016; Li et al., 2018). Fig. S3 shows typical chromatograms obtained from LC–MS/MS analysis of AA-I and AA-II in wheat grain (A and B), maize grain (C and D) and soil (E and F) samples. The chromatographic peaks of AA-I (6.7 min) and AA-II (6.4 min) in the samples were identified by comparing the retention times with those of the standards and by comparing the relative abundance of the quantitative transition to that of the qualitative transition, as described elsewhere. Furthermore, the assay provided an extra criterion for reliable compound identification by comparing the signal intensity of samples with and without the Zn/H^+ treatment. In the sample that was reacted with Zn/H^+, a significantly higher signal intensity should be observed than that in the control that was not treated with Zn/H^+ if the signal was generated from AA (Fig. S1).

The data showed positive identification of AA in 5, 4, and 4 of the collected wheat grain (n = 20), maize grain (n = 20) and soil (n = 20) samples, respectively, with concentrations ranging from sub-μg/kg to μg/kg levels (Table 2). The level is lower than that reported previously

(Chan et al., 2016; Li et al., 2018), which is attributed to the ability of the newly developed method to detect AA at dramatically lower concentrations. Among all the samples that tested positive for AA contamination, AA-I was detected at concentrations significantly higher than those of AA-II. This result is in line with previous observations and could be attributed to the higher concentration of AA-I than that of AA-II in *A. clematitis* weeds, which decayed and released AA into the environment (Chan et al., 2016).

3.4. Application in supermarket flour sample analysis

Previous studies on AA contamination focused on food grain and soil samples collected from rural areas where a high incidence of BEN was recorded. Using the developed method, supermarket wheat ($n = 36$) and maize ($n = 24$) flour samples collected from Serbia and Bulgaria were also tested for the first time. Fig. 3 depicts the chromatograms obtained from LC–MS/MS analysis of AA-I in wheat flour (A) and maize flour (B), showing retention times that are similar to the authentic standards (Fig. 2A). Surprisingly, in 8 out of the 60 tested flour samples, AA were detected at sub-μg/kg levels (Table 2). This may have arisen from wheat or maize grains grown in AA-contaminated areas. In contrast, no AA were detected in the control flour samples collected from supermarkets in Hong Kong, as these sample sources were mostly imported from China, the USA, Australia and Japan.

The results from the study revealed for the first time that nephrotoxic AA not only affect residents in rural areas but also contaminate the food source of citizens in the urban areas of the Balkan Peninsula. Based on these findings, citizens in urban areas are also exposed to nephrotoxic AA through dietary intake and may suffer from AA-associated diseases.

3.5. Comparison with LC–MS/MS and LC-FLD methods

The samples were also analyzed using previously developed LC–MS/MS and LC-FLD methods for comparison (Chan et al., 2003, 2016; Li et al., 2016; Wang & Chan, 2014; Yuan et al., 2007). The results from the analyses were compared with those obtained from the current LC–MS/MS analysis. A comparative view of the AA concentrations in the wheat grain, maize grain, wheat flour, maize flour and soil samples analyzed by LC–MS/MS and LC-FLD methods is depicted in Fig. 4. The analysis showed that the two methods gave similar results ($r^2 = 1.0$) for samples with AA concentrations above the quantification limit of the LC-FLD method (Fig. 4). However, in many of the samples, especially the flour samples obtained from supermarkets, only the newly developed LC–MS/MS method with an enhanced sensitivity can quantitate the AA levels in the samples (Table 2), thus identifying the specific advantage of the new method for the analysis of AA in food products.

4. Conclusion

In conclusion, we developed and validated a novel LC–MS/MS method for the determination of carcinogenic and nephrotoxic AA at trace or ultra-trace levels. One key feature of the method is that treatment with Zn/H^+ dramatically increases the analytical sensitivity and selectivity for AA in ESI-MS (Fig. 1). This method with enhanced sensitivity allows reliable quantification of AA in grain, flour and soil samples containing sub-μg/kg levels of AA, which overcomes one of the major limitations of the current methods for AA identification and allows reliable identification of AA in food samples that are sold in supermarkets in urban areas of the Balkan Peninsula. The results demonstrated for the first time that AA affect not only the previously identified rural population but also residents living in urban areas in countries of the Balkan Peninsula. Emerging evidence shows that AA released from decaying *A. clematitis* weeds are contaminating both the food source and the environment, thus affecting a greater amount of the population; therefore, it is anticipated that the LC–MS/MS method will meet the growing demand for the detection and monitoring of AA in food products and the environment.

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Conflict of interest statement

The authors declare no competing financial or non-financial interest.

Appendix A. Supplementary data

MS parameters for AA and AL analysis on LC − MS/MS; Method validation data from analysing AAs-fortified food grains and flour samples; Schematic illustration of the developed LC − MS/MS method; Chromatograms from LC − MS/MS analyses of AL-I and AL-II, the nitroreduction products of AA-I and AA-II in food grains collected from Serbia. Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.03.073.

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