Contemporary Agriculture, Vol. 73, No. 1-2 (2024): 28-33
DOI: 10.2478/contagri-2024-0004
UDC: 599.731.1

Original scientific paper

OCCURRENCE OF HEAVY METALS (Cd, Pb, As, Hg) IN THE LIVER OF WILD BOARS IN THE REPUBLIC OF NORTH MACEDONIA

ISSN 2466-4774
https://www.contagri.info/

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Submitted: 22.09.2023.
Accepted: 15.12.2023.

SUMMARY

Wild boars, being a common game species, inhabit regions across Eurasia, the southern regions of Asia and certain Indonesian islands. Being omnivorous and due to their relatively long lifespan, wide distribution and a relatively high number of shot animals per annual legal hunting season, they are suitable to be biological indicators of occurrence of heavy metals. Concentrations of cadmium, lead, arsenic and mercury were analyzed by atomic absorption spectrometry and cold vapor atomic absorption spectrometry in the livers of free-living wild boars (n = 58) from two locations in the Republic of North Macedonia. In all four examined heavy metals, no statistically significant difference between the locations was found (p > 0.05). The mean levels of heavy metals were Cd 0.391 mg/kg and 0.339 mg/kg; Pb 0.213 mg/kg and 0.204 mg/kg; Hg 0.034 mg/kg and 0.037 mg/kg; As 0.035 mg/kg and 0.029 mg/kg in the livers of boars from Bitola and Gevgelija hunting sites, respectively. Although some samples (two samples of Hg, ten samples of Cd and two samples of Pb) exceeded the legal limits, the average values for all heavy metals were far below the maximum permitted values. These findings are promising since they suggest that the ecosystem in the Republic of North Macedonia is stable concerning heavy metal pollution.

Key words:
wild boar, liver, Cd, Hg, Pb, As

INTRODUCTION

In the recent decades, industrial production and agricultural activities have caused a significant rise in environmental contamination by heavy metals (Liu, 2003; Bilandžić et al., 2010). In today's world, a variety of toxic substances are polluting all components of the environment, including wild animals (Bilandžić et al., 2009). Environmental pollution sources are not limited to industrial areas, they can also be found in agricultural and other ecosystems, sometimes at considerable distances from the emission sources (Giżejewska et al., 2015; Giżejewska et al., 2017). Exposure to the trace elements, e.g. Cd, Hg, As and Pb, in high amounts is extremely hazardous and can trigger a wide range of adverse effects in both animals and humans (Goyer, 1996).

Biomonitoring is regarded as an essential tool in environmental health assessments. When applied to wildlife, it can serve as an early warning system for assessing potential impact of environmental pollutants like heavy metals on human health (Gómez-Ramírez et al., 2014). Wild game is a good indicator of environmental pollution and it is often used in biomonitoring studies which explore the occurrence of heavy metals (Wolkers et al., 1994). Wild boars (*Sus scrofa*) are recognized as a valuable bioindicator species due to their position at the apex of the food chain, their omnivorous dietary preferences, wide geographical range, sedentary life, relatively long lifespan and for being convenient for sampling through regular hunting activities. In recent years, several studies focusing on heavy metal levels have been conducted on wild boars in European countries including Italy, Poland, Croatia and Spain (Bilandžić et al, 2009; Amici et al., 2012; Mulero et al., 2016; Nawrocka et al., 2020).

Wild boars have a global distribution inhabiting regions across Eurasia, southern regions of Asia and certain Indonesian islands. As omnivores, they have a broad-spectrum diet, including plant components like leaves, roots, seeds, fruits and acorns, as well as invertebrates such as insects and earthworms, vertebrates, cultivated crops (Lee & Lee, 2019; de Souza Reginato et al., 2020) and mushrooms (Brzezicha-Cirocka, 2016). The concentration of potentially toxic elements and various metals in the tissues of wild boars is contingent on their dietary intake of these contaminants primarily sourced from food.

Heavy metals in animals have no biological functions. They enter into the body of wild boars mostly through the food chain (Santiago et al., 1999; Dobrowolska & Melosik, 2002; Lénárt et al., 2023). When taken into the body, heavy metals cannot be destroyed metabolically. The concentration of heavy metals in different organs of wild boars varies, so the largest quantities of Hg and Cd are found in kidneys (Bilandžić et al., 2009; Bilandžić et al., 2010; Berzas Nevado et al., 2012; Mulero et al., 2016; Gašparík et al., 2017; Eltsova & Ivanova, 2021), while Pb is mostly in bones or muscles (Bilandžić et al., 2009; Chiari et al., 2015; Mulero et al., 2016). The data on the occurrence of As is not very conclusive, it occurs in kidneys, livers and bones (Piskorová et al., 2003; Florijančić et al., 2015).

The regulation in the Republic of North Macedonia regarding the maximum levels (ML) of heavy metals in animal organs is fully aligned with the European regulation. According to the Commission Regulation (EC, 2006) no. 1881/2006, Food and Veterinary Agency in North Macedonia established the ML of Cd and Pb in liver of bovine animals, sheep, pigs, poultry and horses, which is 0.5 mg/kg (EC, 2006). The ML of Hg is not specified by this regulation, as the ML of Hg in the Commission Regulation (EC, 2008) no. 149/2008 is 0.1 mg/kg, based on the suggestions of the European Union Reference Laboratory for Chemical Elements in Food of Animal Origin, Rome, (EU-RL CEFAO). The national action level of arsenic in wild boar liver is 0.5 mg/kg in accordance with the Annual Plan (2020) for monitoring residues and illegal substances in live animals, products and raw materials of animal origin for 2020.

In the Republic of North Macedonia, to our knowledge, no previous research has investigated the occurrence of heavy metals in game meat and organs. North Macedonia as a country has been quite unexplored in this regard. This is practically the first research aimed at determining the concentrations of heavy metals in game tissues. Similar studies have been carried out in several countries on the territory of Europe, including neighboring countries such as Croatia, Serbia, Bulgaria, etc. (Florijančić et al., 2015; Beuković et al., 2022). The primary objective of this study was to provide new data on the occurrence of the environmental contaminants Pb, Cd, As and Hg in liver samples of wild boars shot during regular hunting in the hunting grounds within Bitola and Gevgelija municipalities.

MATERIAL AND METHODS

Study area

The study area included a total of 10 hunting grounds covering the territory of the municipalities of Bitola (4 hunting grounds) and Gevgelija (6 hunting grounds). One of the reasons to choose these two locations is their geographical distance to ensure that wild boars from one location could not easily reach the other. Another reason is that there is a potential larger-scale polluter in Bitola region, while there is none in Gevgelija. The city of Bitola is located in the southwestern part of North Macedonia, at the northeastern foot of Baba mountain and the southeastern foot of Oblakovska mountain, in the central part of Pelagonian Valley. Only 12 km east of the city of Bitola, among the villages Suvodol, Paralevo, Vranjevci, Biljanik, Novaci and Aglarci, there is Bitola Mining and Energy Plant (REK) comprising Thermal Power Plant (846,453 m^2), Suvodol Mine (26,880,893 m^2), Brod-Gneotino Mine (9,612,654 m^2) and Ash Landfill (1,107,163 m^2). The main activity of the complex is production of electricity. It consists of Thermal Power Plant Bitola I, II and III (three blocks) with input thermal power of 659 MWh for each block, i.e. total installed power of 1,977MWh, and it provides an average annual production of 3.5 million MWh of electric energy. Coal mining from Suvodol and Brod-Gneotino mines and ash and slag waste disposal in the Landfill are also carried out in REK Bitola.

Sampling

In the period between 2017 and 2021, a total of 58 liver samples of wild boars (40 males and 18 females) were collected from two locations in North Macedonia over the regular hunting season. The sampling procedure excluded parts damaged by bullets, i.e. all samples were without any impact damage (Dobrowolska & Melosik, 2008; Danieli et al., 2012). The samples were individually packed, marked and frozen at -20 degrees until the laboratory analysis procedure. There were no wild boars that were shot for the purpose of conducting this research - all samples were collected by hunters during the regular hunting season.

Analysis

Equipment and sample analysis

The analysis of Pb, Cd, Hg and As was performed by applying atomic absorption spectrometry. Electrothermal atomic absorption spectrometer (ET-AAS) model AAnalyst 600 (Perkin Elmer, Waltham, Massachusetts) was used for Pb, Cd and As, and cold vapor atomic absorption spectrometer (CV-AAS) model FIMS 100 (Perkin Elmer, Waltham, Massachusetts) for analysis of Hg. Data acquisition and calculations were performed by WINLAB 32 software for AA, version 6.2.0.0079 (Perkin Elmer, Waltham, Massachusetts). Sample preparation was performed by pressure digestion, using high-performance microwave digestion system Ethos Up (Milestone Srl, Sorisole, Italy), according to EN 13805:2002 (CEN, 2002a). Approximately 1 g of homogenized sample (with accuracy of 0.01 g) was weighed in Teflon tube, and 5 mL 67 % nitric acid for atomic absorption (Merck, Darmstadt) and 1 mL of 30 % hydrogen peroxide (Merck, Darmstadt) were added. The sample was mineralized following the prescribed pressure and temperature program (CEN, 2002a). The mineralized and cooled sample was transferred into 25 mL volumetric flask and filled up with double demineralized water. Analysis of Pb, Cd and As was performed according to EN 14084:2003 (CEN, 2003) following the prescribed temperature and time programs at selected wavelengths. Hg analysis was performed according to EN 13806:2002 (CEN, 2002b), using tin-(II) chloride solution in HCl as reducing agent. For instrument calibration, we used suitably diluted solutions of certified reference materials (CRM) of Pb, Cd, As and Hg (all purchased from Carl Roth GmbH, Karlsruhe, Germany).

Method performance characteristics and quality control

The method quality assurance was performed according to the requirements laid down in Commission Regulation (EC, 2007). The method validation was performed by analysis of CRM – offal liver FAPAS test material 07199. The method performance characteristics were the following: linearity from 5 calibration points was in the range 3.716-50 µg/L, 0.319-6 µg/L, 0.018-20.0 µg/L, and 0.014-5.0 µg/L for Pb, Cd, As and Hg, respectively, with $R^2 > 0.99$; limits of detection values were from 0.014 up to 3.716 µg/L, and for limits of quantification from 0.045 up to 12.390 µg/L; the method precision (RSD) ranged from 4.98 to 9.94 % (n=6), and method accuracy from 91.10 to 104.25 % (n=6). The determined method performances were in line with the requirements laid down in the respective regulative (EC, 2007). Internal quality control was performed by analyzing the above-mentioned CRM with every batch of samples. The method was accredited according to EN ISO/IEC 17025:2017, and external quality control was provided by multiple satisfactory results in proficiency tests provided by FAPAS-FERA (UKAS accreditation) and the European Union Reference Laboratory for metals, EURL-MN, DTU (DANAK accreditation).

Statistical analysis

Statistical analysis was performed using Statistica software version 12.5.192.7 (StatSoFT STATISTICA Software) for descriptive analysis and Microsoft Excel 2016 MSO (16.0.4312.1000) for analysis of variance. Descriptive statistical data were calculated and presented as mean, maximum and minimum by location. Differences between sampling areas were examined using one-way analysis of variance (ANOVA) test. All statements of significance were based on 0.05 level of probability ($p \leq 0.05$).

RESULTS AND DISCUSSION

The results of the analysis for Hg, Cd, As and Pb concentrations in wild boar livers are shown in Table 1. The analysis of the four elements indicated no statistically significant difference between Bitola and Gevgelija locations ($p > 0.05$). The mean concentration of Hg in the livers was 0.034 mg/kg in Bitola and 0.037 mg/kg in Gevgelija. The maximum concentration from both locations was 0.169 mg/kg found in a sample from Gevgelija location. This concentration exceeded the ML prescribed by the legislation of North Macedonia. Although there were samples that exceeded the ML, the mean value was within the legislation. It should be also noted that a total of 6 samples in both locations were below the limits of detection and quantification. Similar results for the mean concentrations of Hg in livers were reported by Dobrowolska & Melosik (2002), Berzas Nevado et al. (2012) and Durkalec et al. (2015).

However, our results were lower compared to the results obtained in Russia by Eltsova & Ivanova (2021), where the mean value for Hg in wild boar livers was 0.419 mg/kg**.** On the other hand, the results in our study for Hg concentration were two times higher compared to the results obtained in Poland by Nawrocka et al. (2020), but they were still within the ML prescribed by the legislation.

Regarding the concentration of Cd in both locations, only two samples (3.4%) were below the detection limit. Satarug et al. (2003) reported that Cd can be accumulated, and once absorbed it is rapidly cleared from the blood and concentrated in various tissues, especially in the main target tissues, kidneys and liver, where it binds to metallothionein. Only ten samples (17.8%) had concentrations higher than the ML, but the mean value of Cd concentration was 0.391 mg/kg for samples from Bitola and 0.339 mg/kg from Gevgelija, both of which are within the ML provided by the legislation. Compared to other research, our results correspond to the results obtained by Santiago et al. (1998) in Spain, Bilandžić et al. (2009) in Croatia and Gašparík et al. (2017) in Slovakia. The average level of Cd in wild boar livers from Upper Silesia (southwestern part of Poland) was much higher (5.573 mg/kg) (Durkalec et al., 2015) compared to our study, but the reason might be that pollution by toxic metals in this area is considered to be the highest in Poland (Bielińska & Mocek-Płóciniak, 2010; Pajak & Jasik, 2011). In Croatia, Italy and Spain, the values for Cd concentration found in wild boar livers were several times lower than in our research (Amici et al., 2012; Danieli et al., 2012; Crnić et al., 2015). The maximum concentration of Cd found in both locations was 1.203 mg/kg in a sample from Bitola, which is 2.5 times higher than the ML adopted by the national legislation.

Table 1. Concentrations of Hg, Cd, As and Pb (mg/kg) in wild boar livers in two locations in North Macedonia

Elements	Location	N	Mean±SD	Median	Minimum	Maximum
Mercury (Hg)	GV	26	0.037 ± 0.034	0.028	0.0026	0.169
	BT	26	0.034 ± 0.035	0.025	0.0045	0.137
Cadmium (Cd)	GV	28	0.339 ± 0.197	0.323	0.046	0.710
	BT	28	0.391 ± 0.275	0.342	0.037	1.203
Arsenic (As)	GV	26	0.029 ± 0.026	0.024	0.001	0.122
	BT	23	0.035 ± 0.020	0.032	0.002	0.073
Lead (Pb)	GV	26	0.204 ± 0.135	0.182	0.039	0.622
	BT	24	0.213 ± 0.228	0.137	0.021	1.095

Legend: GV - Gevgelija; BT - Bitola; N - number of samples; SD - Standard deviation

The mean concentration of As in the livers was 0.035 mg/kg in Bitola and 0.029 mg/kg in Gevgelija. Both average values were below the ML, and even the maximum value of 0.122 mg/kg found in the sample from Gevgelija location did not exceed the ML prescribed by the national regulation.

The results for Pb concentration obtained in this study were similar to the previously described concentrations of heavy metals, with no statistically significant difference between locations. Eight samples (13.8 %) were below the limit of detection. In addition, only two samples exceeded the ML, although one sample from Bitola location was two times higher than the ML. As comparisons are sometimes difficult to be carried out due to differences in data manipulation, our results for Pb concentration fall within the ML prescribed by legislation. The mean Pb concentrations in liver (0.204 mg/kg in Gevgelija and 0.213 mg/kg in Bitola) were 12 to 13 times lower than levels (2.61±8.35 mg/kg) found in Spain by Santiago et al. (1998), almost 10 times (1.919 mg/kg) and two times lower (0.391 mg/kg) compared to those found in two locations in Spain by Reglero et al. (2009), but similar to findings reported by Mulero et al. (2016) (0.189 mg/kg) also in Spain. Slightly lower Pb concentrations in wild boar livers compared to our results were determined in Croatia (0.111 mg/kg) and Slovakia (0.118 mg/kg) (Bilandžić et al., 2009; Gašparík et al., 2017).

Over the last thirty years, industrial production in the Republic of North Macedonia has considerably declined, which is probably reflected in the emission of heavy metals in the environment. Also, the content of As, which occurs naturally in certain regions, is not alarming and there are no data or any indication of a problem in North Macedonia. Most of the studies that we compared our results with were conducted in the areas around former or current industrial plants, and some of these studies were carried out in the most industrially polluted areas in those countries. This may be one of the reasons for the differences in the results compared with other studies, especially in cases where the differences were significant with tens of times higher values compared to the results obtained in this study.

CONCLUSION

This is one of the first studies on the occurrence of heavy metals in edible organs (liver) of wild boars in the territory of the Republic of North Macedonia. Wild boars, with their biological characteristics, are a very good bioindicator of

pollution. Although some samples (two samples of Hg, ten samples of Cd and two samples of Pb) exceeded the ML, the average values for all heavy metals were below the ML. There was no statistically significant difference in the occurrence of any heavy metal depending on the location. These results are encouraging because they indicate a relatively good condition of the ecosystem in terms of heavy metal pollution, especially taking into account the location of Bitola, where there is one of the largest thermal power plants in the Republic of North Macedonia. In future studies, more extensive research needs to provide a clearer picture of the occurrence of heavy metals in North Macedonia.

Conflict of interest: The authors declare that they have no conflict of interest.

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