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COMPARATIVE HYGIENIC ASSESSMENT OF THE BEHAVIOR AND RESISTANCE OF PESTICIDES IN THE SOILS OF SOUTH-EAST EUROPE WHEN USING FORMULATIONS WITH 3RIVE 3D TREATMENT TECHNOLOGY

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Key words: hazardous pesticides, maximum allowable level, contaminated food products, human health, half-life period.

Abstract

Undoubtedly, one of the important factors that allows to reduce the pesticide load on target and non-target objects is the method of chemical plant protection products (CPPPs) application. The 3RIVE 3D pesticide application system is an innovative technology for plant treatment that allows you to reduce the rate of pesticides used and increase their effectiveness.

After the application of Brigade 3Rive 3D, SC formulation using the innovative 3Rive3D technology (soil application with simultaneous sowing of corn seeds) found that on the day of treatment, the level of bifenthrin in the soil was <0.05 mg/kg. On the 3rd day after treatment, the amount of bifenthrin in the soil slightly increased and amounted to 0.083 **±0.015** mg/kg, on the 7th day the concentration of bifenthrin was 0.06 **±0.01, which, in turn, is less than the level of the** recommended maximum allowable level (0.1 mg/kg).

It was established that when applying the Brigade 3Rive 3D, SC formulation using 3Rive3D technology, the half-life of bifenthrin was 3.6 days, which is lower than the average values obtained in other countries of the European region.

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Introduction

Numerous scientific studies (Antonenko et al. 2023, Syafrudin et al. 2021, Lamichhane 2021, Tkachenko et al. 2020, Arias-Estévez et al. 2008) have shown that the long-term pesticides application leads to their accumulation in the soil, migration into groundwater and atmospheric air. Scientific studies in different countries confirm that the soil is an important link in the migration of pesticide compounds, and its degree of contamination with xenobiotics affects the food products safety. The amount and percentage of pesticides entering agricultural crops depends on the physical and chemical properties of the soil, agro-climatic conditions, the application method and the formulations' application rates.

Undoubtedly, one of the important factors that allows to reduce the pesticide load on target and non-target objects is the method of chemical plant protection products (CPPs) application (Melnichuk et al. 2022).

According to Directive 2009/127/EC 2009 the use of machines and technologies for applying pesticides is regulated. One of the main aspects considered in this directive is the risk assessment of the machinery use for the pesticides' application. To ensure the safety of people and the environment, additional requirements have been introduced for machinery used to apply pesticides. In particular, the minimization of losses on non-target areas and the provision of maximum pesticides sedimentation on target objects are regulated. These requirements contribute to reducing the pesticides application rates, increasing the efficiency of their use, and reducing the negative impact on the environment (Borysenko et al. 2023).

The aim is comparative hygienic assessment of the behavior and resistance of pesticides in the soils of south-east Europe when using formulations with 3RIVE 3D treatment technology.

Materials and Methods

The 3RIVE 3D pesticide application system is an innovative technology for plant treatment that allows you to reduce the rate of pesticides used and increase their effectiveness. 3RIVE 3D technology converts the traditional high-volume application technology into a low-volume one thanks to the patented technology (Borysenko et al. 2023). 3RIVE 3D is an innovative insecticide delivery platform developed by FMC and Micro-Trak Systems Inc.

An experimental applicator 3D RIVE RESEARCH MACHINERY (Micro-Trak Systems, Inc.) was mounted on the "Universal dotted seeder" planter of precision seeding UPS-4 with inter-sectional placement of wheels aggregated with a tractor. Cultivators treated the soil with simultaneous sowing of corn on an area of 1 ha. The formulation was used at an application rate of 1.2 l/ha. The rate of the working solution is 2.5–3.0 l/ha. The formulation is mixed with water and air to form a voluminous foam into which the seed is placed during sowing.

The study of the bifenthrin behavior was carried out using a specific hygienic method of a natural experiment, and the plant and soil sampling was carried out according to applied rules, starting from the first day of treatment, and subsequently at regular intervals during the crop vegetation period until harvesting. The last sampling was carried out at the harvest. In parallel, the selection of crops control samples, plants' green mass and soil was carried out in order to compare them with the results of the treated target objects.

Table 1 presents data on the conditions and place of the studied formulation application and its active ingredient using modern plant protection technologies.

The xenobiotic persistence index (XPI) was calculated according to the formula given in (Li 2022):

$$\mathrm{XPI} = \tau_{95} \cdot \ln \frac{\mathrm{P}_m}{\mathrm{MAL}}$$

where:
XPI – is the xenobiotic (pesticide) persistence index,
τ_{95} – pesticide half-life period (average or for specific conditions) [months];
MAL – maximum allowable level of pesticide in soil [mg/kg],
P_m – the maximum recommended dose of pesticide [kg of active substance/ha].

We used a scale of values for the XPI values evaluation, according to which the XPI is less than 5 – the level of soil pollution is assessed as safe, from 5–20 – moderately dangerous, 20–60 – dangerous, with values greater than 60 – very dangerous (Li 2022).

Table 1
Characterization of the soils of the regions, where agricultural land was treated with the use of innovative pesticide application technologies (PANAGOS et al. 2011)

Place of treatment	Province, climatic zone	Soil type
Kyiv region	Right Bank Province, forest steppe	chernozems are podzolized mainly on loess rocks
Zhytomyr region		meadow and chernozem-meadow soils + turf-medium and slightly podzolic sandy and loamy soils
Vinnytsia region		typical chernozems are moderately highly humus-accumulative
Kyiv region	Left Bank Lowland Province, forest steppe	dark gray podzolized, moderately weakly humus-accumulative + chernozems, moderately humus-accumulative, podzolized

Results and Discussion

Bifenthrin practically does not migrate along the profile, both in sandy and soils rich in organic compounds (RAMASUBRAMANIAN et al. 2021). The compound is relatively poorly soluble in water; therefore, the substance does not concentrate in aquifers. The half-life period in the soil ranges from 7 days to 8 months, depending on the type of soil (RAMASUBRAMANIAN et al. 2021).

Laboratory studies in the EU: τ_{50} range 54.2–173.7 days, τ_{90} range 223–577 days, field studies: τ_{50} range 5.4–267 days, τ_{90} range 135.3–965.2 days. Other studies: τ_{50} range from 65 to 125 days or range from 2 to 6 months depending on the type of soil (RAMASUBRAMANIAN et al. 2021).

We conducted field studies in different agro-climatic zones of south-east region of Europe with different types of soil, for a more detailed study of the behavior of the Brigade 3Rive 3D, SC active ingredients in environmental objects when it is applied using 3RIVE 3D technology.

The results of determining the level of residual amounts of the studied active substances are shown in Table 2.

Table 2

The content of bifenthrin in soil samples when using the Brigade 3Rive 3D, SC formulation

Standard, MAL	Content [mg/kg] in the soil in the treatment zone due to:			Re-entry intervals [days]	
	1 hour	3 days	7 days	mechanized works	manual works
0.1	<0.05	0.083 ±0.015	0.06 ±0.01	does not require	does not require

Explanations: MAL – maximum allowable level of pesticide in soil [mg/kg]

After the application of Brigade 3Rive 3D, SC formulation using the innovative 3Rive3D technology (soil application with simultaneous sowing of corn seeds) found that on the day of treatment, the level of bifenthrin in the soil was <0.05 mg/kg. On the 3rd day after treatment, the amount of bifenthrin in the soil slightly increased and amounted to 0.083 ±0.015 mg/kg, on the 7th day the concentration of bifenthrin was 0.06 ±0.01, which, in turn, is less than the level of the recommended MAL (0.1 mg/kg).

The obtained levels of the content of pesticide active ingredient residual quantities in the soil allowed us to calculate the destruction rate constants (k) and quantitative parameters of stability in environmental objects by the method of least squares: periods of decay for 50, 95 and 99% (τ_{50}, τ_{95} and τ_{99}) – Table 3.

Table 3

The bifenthrin degradation rate in soil (BORYSENKO et al. 2023)

Indices of degradation rate in soil [days]				
k^{-1}	τ_{50}	τ_{95}	τ_{99}	τ_{50}*
0.194 ±0.031	3.55 ±0.965	15.38 ±2.134	26.66 ±4.64	26.0–86.8

Explanations: k^{-1} – the destruction rate constant; τ_{50} – the period of 50% of the substance initial amount decomposition; τ_{95} – the period of 95% of the substance initial amount decomposition; τ_{99} – the period of 99% of the substance initial amount decomposition; * – according to the literature

When applying Brigade 3Rive 3D, SC formulation using 3Rive3D technology, the bifenthrin's τ_{50} was 3.6 days, τ_{95} – 15.4 days and τ_{99} – 26.7 days, the destruction rate constant was equal to 0.19, which is lower than the average values obtained in other countries of the European region. In our opinion, this is explained, first, by the feature of the 3Rive3D technology, which allows to reduce the pesticide consumption rate as much as possible and ensures its accurate introduction into the furrow simultaneously with the sowing of corn seeds. Also, climate and weather conditions, type of soils, their pH value, air humidity and temperature, intensity of ultraviolet radiation, and others are important.

According to State Standard (*Pesticides. Classification…* 8.8.1.002-98), by to the stability indices in the soil, bifenthrin can be classified as a low-hazard compound (hazard class 4).

The results of field studies using the innovative technology of pesticide application with 3Rive3D technology in the agro-climatic conditions of south-east Europe indicate its lower persistence under the conditions of these treatments compared to the values established in the soils of other countries.

We also calculated the Xenobiotic Persistence Index (XPI) in the soil, since the τ_{95} of bifenthrin applied by us in an innovative way differs from the values established in other countries. The calculation and evaluation of the xenobiotic persistence index in the soil will allow establishing the risk of cumulation and the degree of soil contamination with pesticides.

According to the results obtained during field studies when applying the Brigade 3Rive 3D, SC formulation in the innovative application technology (3Rive3D technology) in different soil and climatic conditions of south-east Europe, the average values of bifenthrin τ_{95} was (15.4 days) 0.51 months. Tentatively allowable concentration in soil of bifenthrin is 0.1 mg/kg. The maximum recommended consumption rate for the pesticide application recalculated for bifenthrin is 0.23 kg/ha. Hence,

$$\mathrm{XPI}_{\mathrm{bifentrin}} = 0.51 \cdot \ln\frac{0.23}{0.1} = 0.43.$$

The obtained XPI value of bifenthrin (0.43) made it possible to classify the studied active ingredient as a pesticide with a safe potential level of soil contamination (XPI <5) and characterizes it as a short-lived compound in the studied soil and climatic of conditions of south-east Europe when applying formulations based on it using 3Rive3D technology. The assessment of the XPI value in the soil allowed us to predict a relatively low expected level of accumulation and soil contamination with pesticides (BORYSENKO et al. 2022).

Conclusions

1. It was established that when applying the Brigade 3Rive 3D, SC formulation using 3Rive3D technology, the half-life of bifenthrin was 3.6 days, which is lower than the average values obtained in other countries of the European region. According to State Standard (*Pesticides. Classification…* 8.8.1.002-98), the compound can be pertained to the 4^{th} class of danger (low-dangerous compounds) based on stability indices in the soil.

2. The obtained value of the persistence index (XPI) of bifenthrin (0.43) made it possible to attribute the studied active ingredient to pesticides with a safe potential level of soil contamination and characterizes it as a short-lived compound in the studied conditions when applying formulations based on it using 3Rive3D technology.

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EFFECT OF DIETARY CHOLINE ON THE PRODUCTION PERFORMANCE AND CARCASS CHARACTERISTICS OF COBB 500 CHICKENS

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Key words: broiler chicken, growth performance, carcass characteristics, choline.

Abstract

Choline serves several crucial metabolic functions, making it an essential component in poultry diets which include lipid transport, cell signalling, and biosynthesis of methylated compounds. The objective of this study was to evaluate the performance and carcass characteristics of the Cobb 500 chickens fed dietary choline. One hundred- and forty-four-day-old (42.16 ±0.15), unsexed Cobb 500 chickens were randomly assigned to three treatment groups with four replications of twelve (12) chicks each in a Completely Randomized Design. Three levels of choline (0, 1200, 1400 ppm/100 kg of feed) were supplemented in the chick's feed at the starter phase (0–21days), while at the finisher phase (21–49 days) three levels of choline (0, 800, 1000 ppm/100 kg feed) were also supplemented in the chicken's feed. Results showed that different levels of choline had no significant effect ($P > 0.05$) on the weight changes of broiler chickens at the starter and finisher phases. However, the feed conversion ratio was best ($P < 0.05$) for chicks supplemented with 1200 ppm at the starter phase compared to the finisher phase. At the finisher phase, feed intake (3216.93 g/bird) of birds offered 800 ppm choline were significantly ($P < 0.05$) reduced when compared to control diets (3380.11 g/bird). Dressing percentage significantly ($P < 0.05$) increased at 800 ppm while the thigh decreased ($P < 0.05$) with choline increment. In conclusion, choline supplementation in the diets of broiler chickens at 1200 and 800 ppm/100 kg feed impro-

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ved the growth performance and carcass characteristics of Cobb 500 broiler strain chickens at starter and finisher phases respectively.

Introduction

The nourishing elements in food that an organism needs to grow and survive are known as nutrients. Micronutrients supply the cofactors required for metabolism to occur, while macronutrients supply the bulk of the energy needed for an organism's metabolic system to work. Both micronutrients and macronutrients can be sourced from the environment.

Choline serves several crucial metabolic functions, making it an essential component in poultry diets which include lipid transport, cell signalling, and biosynthesis of methylated compounds. Choline is required for the formation and maintenance of cell membranes and organelles such as mitochondria and microsomal, as well as the appropriate maturation of the bone cartilage matrix (ARELE et al. 2015). Unlike other vitamins, choline can be synthesized through de novo synthesis, but the inability to synthesize enough choline can cause choline deficiency, resulting in growth retardation and perosis in young chicks. According to LIN et al. (2020), choline deficiency is often linked to fatty liver development due to its function in lipid metabolism, and which has been demonstrated in chicken models. Moreover, the bioavailability of choline varies largely and depends on raw material sources and bird related factors such as type, strain, age, feed consumption, dietary crude protein, and methionine (NRC 1994).

Recent studies have shown mixed outcomes regarding the effect of dietary choline supplementation when added above the amount that is naturally present in maize and soybean meal-based diet in broiler growth performance (MCDOWELL and WARD 2008, NORVELL and NESHEIM 1969). While current choline recommendations may be sufficient to prevent deficiency, they are not necessarily adequate for optimizing the growth performance and carcass yield of broilers. Therefore, the objective of this study was to evaluate growth performance and carcass characteristics of Cobb 500 broiler strain fed on dietary supplementation of choline grown from 0 to 49 days of age.

Materials and Methods

Experimental site

The experiment was carried out at the Poultry Unit of the Directorate of University Farms (DUFARMS), Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. The farm is located at latitude 7.15ºN, longitude 3.26ºE. The site is 76 m above sea level in the tropical forest vegetation zone with an average temperature of 30.19ºC and relative humidity of 82%.

Experimental design and treatment

A total of one hundred and forty-four day-old unsexed broiler chickens (Cobb 500) were procured from a reputable hatchery in Ibadan, Oyo state Nigeria. On arrival, the chicks were weighed and were randomly assigned to three treatment experimental groups equally with four replications of twelve chicks each in a Completely Randomized Design. Each replicate was raised in a pen measuring $3m^2$ with wood shavings as bedding in a tropical climate. Chicks assigned to Treatment 1 belonged to the control group with no choline supplemented into their feed, those in Treatment 2 had 1200 ppm of choline supplemented into their feed and those in Treatment 3 had 1400 ppm of choline supplemented into their feed at the starter phase (0–21days), while at finisher phase (22–49 days), chicks assigned to treatment 2 had 800 ppm of choline supplemented into their feed and those in treatment 3 had 1000 ppm of choline supplemented with into their feed. The feed was introduced to the day–old chicks on the day of arrival to the last day of the experiments (49 days), they were given fresh clean water *ad libitum* throughout the experiment. The chicks were vaccinated against Newcastle disease and Infectious Bursal disease.

Experimental dietary composition

Choline was procured from a reputable animal feed store in Abeokuta, Ogun State. Three inclusion levels of choline were administered for the starter phases (0, 1200 and 1400) ppm and at finisher phases, three levels of choline were also administered (0, 800 and 1000) ppm for the experimental diets' composition of the broiler chickens as shown in Table 1. The inclusion level was to meet the nutritional composition of broiler chickens at starter and finisher phases.

Table 1

Composition [%] of experimental diets for starter and finisher phases of broiler chickens

Feed ingredients	Starter	Finisher
Maize	52.00	58.40
Soybeans meal	18.00	10.00
Fish meal (72%)	2.20	1.00
Groundnut cake	17.50	14.00
Wheat offal	4.30	10.60
Bone meal	3.00	3.00
Limestone	2.00	2.00
Choline free premix	0.25	0.25
Methionine	0.25	0.25
Lysine	0.25	0.25
Salt (NaCl)	0.25	0.25
Total [%]	100	100
Determined analysis		
Crude protein [%]	22.46	18.15
Crude fibre	3.61	3.51
Ether extracts	4.04	3.88
Metabolisable energy [MJ/kg]	11. 87	12.14

Explanations: choline free premix/vitamins: vitamin A – 8700 IU; vitamin D3 – 2300 IU; vitamin E – 16 IU; vitamin B12 – 31 mg; riboflavin – 6.6 mg; niacin – 28 mg; Ca panthothenate – 35 mg; menadione – 1.50 mg; folic acid – 0.80 mg; thiamine – 3 g; pyridoxine – 2.5 mg; biotin – 30 mg; ethoxyquin – 125 mg; Mn – 80 mg; Zn – 75 mg; Fe – 50 mg; Cu – 10 mg, I – 1 mg

Data collection

Growth performance

Data on body weight (BW) and feed intake (FI) were collected weekly at the starter and finisher phases and were used to calculate average daily gain (ADG) and feed conversion ratio (FCR).

$$\text{Feed intake [g]} = \frac{feed\ of\ ferd\ -\ feed\ leftover}{number\ of\ birds}$$

$$\text{Weight gain per bird [g]} = \frac{final\ weight\ -\ initial\ weight}{number\ of\ birds}$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{total\ feed\ intake}{total\ body\ weight\ gain}.$$

Carcass yield determination

At the end of the experiment (49 days), two broilers were randomly selected per replicate, weighed, slaughtered, de-skinned and eviscerated. The eviscerated carcass was weighed and the head and shank were removed to record the dress weight. The cut-up parts and the organs such as the thigh, drumstick, breast, back, spleen, liver, gizzard, heart, bursa and thymus were weighed with a digital scale. The dress percentage was expressed as a percentage of the live weight.

$$\text{Dress percentage [\%]} \ \frac{dressed\ weight\ [g]}{live\ weight\ [g]} \cdot 100$$

Statistical analysis

All data collected during the experimental period were subjected to One Way Analysis of Variance (ANOVA) using a Completely Randomized Design in accordance with SPSS (2009) and Duncan's multiple range tests were used to reveal significant differences at $p < 0.05$ among the treatment means.

Model

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$

where:
Y_{ij} – the observed value of j_{th} animal fed i_{th} treatment,
μ – population mean,
T_i – effect of i_{th} treatment,
ε_{ij} – random error.

Results

Growth performance

The growth performance of broiler chickens fed choline is presented in Tables 2 and 3, respectively. At the starter phase, there was a significant ($P < 0.05$) difference in the feed conversion ratio (FCR). The best FCR of 0.91 was obtained in birds on T2 (1200 ppm/100 kg of feed) compared to the value of 0.99 obtained for birds on T3 (1400 ppm/100 kg of feed) and T1 (control). On the 49th day (Table 3), a significant ($P < 0.05$) difference was obtained in the feed intake. Chickens fed with choline at an inclusion level of 800 ppm/100 kg (T2) recorded the lowest feed intake (3216.93 g/birds) while the highest feed intake (3380.11 g/birds) was obtained in chickens fed with 0 ppm/100 kg of feed (T1).

Table 2

Starter phase growth performance of Cobb 500 chickens fed dietary choline

Inclusion levels of choline [ppm]				
Parameters	T1 (0)	T2 (1200)	T3 (1400)	SEM
Initial weight [g/bird]	42.00	42.33	42.15	0.14
Final weight [g/bird]	400.19	448.96	416.67	9.76
Weight gain [g/bird]	358.19	406.62	374.52	9.70
Daily weight gain [g/bird/day]	17.06	19.363	17.84	0.46
Feed intake [g/bird]	370.05	371.06	364.33	6.01
Feed conversion ratio	1.04^{a}	0.91^{b}	0.99^{ab}	0.02

Explanations: *a,b* – means in a row with different superscripts are significantly different ($P < 0.05$); SEM – Standard error of mean

Table 3

Finisher phase growth performance of Cobb 500 chickens fed dietary choline

Inclusion levels of choline [ppm]				
Parameters	0	800	1000	SEM
Initial weight [g/bird]	400.19	448.96	416.67	9.76
Final weight [g/bird]	1587.50	1550.00	1560.00	15.48
Weight gain [g/bird]	1187.31	1101.04	1143.33	22.17
Daily weight gain [g/bird/day]	42.40	39.32	40.83	1.68
Feed intake [g/bird]	3380.11^{a}	3216.93^{b}	3238.74^{ab}	32.78
Feed conversion ratio	2.85	2.92	2.83	0.08

Explanations: ^{a,b} – means in a row with different superscripts are significantly different ($P < 0.05$); SEM – standard error of mean

Carcass characteristics

The result of the carcass yield of Cobb 500 chickens fed diets supplemented with choline is presented in Table 4. There was no significant ($P > 0.05$) difference in the live weight, eviscerated weight and internal organs. However, the chickens reared on T1 (0 ppm choline) and T2 (800 ppm choline) had higher ($P < 0.05$) dressing percentages than those in T3 (1000 ppm choline). The result also revealed that there was decrease in thigh weight ($P < 0.05$) as the choline level increases.

Table 4

Carcass characteristics of Cobb 500 chickens fed dietary choline

Inclusion levels of choline [ppm]				
Parameters	T1(0)	T2(800)	T3(1000)	SEM
Live weight [g]	1587.50	1550.00	1560.00	48.94
Eviscerated weight [g]	1084.75	1045.75	980.25	39.33
Dressing percentage	56.47^{a}	56.57^{a}	51.58^{b}	0.96
Breast [%]	17.27	17.33	16.52	0.32
Back [%]	13.52	13.09	11.94	0.35
Thigh [%]	10.41^{a}	9.12^{b}	8.72^{b}	0.27
Drumstick [%]	9.53	9.80	8.88	0.21
Spleen [%]	0.11	0.12	0.13	0.011
Liver [%]	2.12	2.81	2.24	0.28
Gizzard [%]	2.02	1.80	1.89	0.063
Heart [%]	0.56	0.45	0.48	0.024
Bursa [%]	0.056	0.080	0.090	0.014
Thymus [%]	0.20	0.058	0.29	0.050

Explanations: ^{a,b} – means in a row with different superscripts are significantly different ($P > 0.05$); SEM – standard error of mean

Discussion

The similarity observed in the body weight and daily weight gain is in accordance with the findings of SAARINEN et al. (2000) and RAFEEQ et al. (2011) who reported that dietary supplementation of choline had no significant ($P > 0.05$) effect on the body weight and daily weight gain of broiler chickens in a 28-day experimental period. This also agrees with the study of GREGG et al. (2022), who reported that body weight and BW gain were not impacted by supplemental choline chloride in the diet of broiler chickens fed at different inclusion levels in a 66 days experimental period. In the present study, the weight gain means were comparable, but the feed conversion is best in supplemented groups. This disparity observed might be hinged on several factor like broiler's species used, location, ingredient's composition of the diets etc. Though significantly similar, chickens fed a diet with choline performed better (numerically higher value) at the starter phase as supported by the report of PESTI et al. (1980) and SONBOL (1990) that weight gain of broiler chicks at the starter phase increased as dietary choline elevates to 1900 mg/kg. The similar gain observed at the finisher phase might be that the supplemented choline was used for other physiological functions rather than growth.

The feed conversion ratio of the chicks supplemented with dietary choline was best at starter phase (0–21 days) in the present study. The similar weight gain observed in this study corroborates the earlier report of EMMERT and BAKER (1997) who stated that animals do require choline in feed to maintain body physiological functions rather than growth. The similar feed conversion ratio observed at the finisher phase (22–49 days) agrees with the report of ALAGAWANY et al. (2016) who observed a similar feed conversion ratio during 21–42 days of age of Cobb 500 broiler chickens. The similar feed conversion ratio observed was hinged on the fact that the chloride in the choline chloride might have disturbed the ion balance resulted in lower feed conversion ratio (ALAGAWANY et al. 2016). In contrary to the current result, SUMMER et al. (2013) indicated no positive effects of dietary choline on broiler feed intake during the last three weeks of rearing. Based on weight changes EBAHIMNEZHAD et al. (2011) also reported that dietary choline at different inclusion levels had no significant effect on body weight in poultry in contrast to the feed intake. However, based on the present study, the feed conversion ratio of broiler chickens supplemented with choline at 21–49 days shows that the birds can still maintain the weight gain while reducing feed consumption of broiler chickens if reared for a longer period. This can be made possible because of the choline in various physiological processes like, metabolism (choline is converted to betaine, it is involved in methylation reactions, influencing energy metabolism and nutrient utilization (ALAGAWANY et al. 2022), lipid transport (choline is a precursor to phospholipids which are essential for lipid transport and membrane structure), gene expression (choline affects gene expression and this influences various biological processes such as growth and development) (IGWE et al. 2015, LI et al. 2023) and gut health (choline can influence the gut microbiome, which plays a crucial role in nutrient absorption and utilization) (ABRAMOWICS et al. 2020, GOH et al. 2021).

The prevention of accumulated fat in the hepatocytes or the development of fatty liver is one of the benefits of choline in poultry production (WORKEL 2004). In the present study, the supplementation of choline to the dietary feed of the broiler chickens have no significant effect ($p > 0.05$) on the thigh, drumstick, breast, back, spleen, liver, gizzard, heart, bursa and thymus. This is in accordance with (ALAGAWANY 2015) who reported that the various levels of choline do not show any consistent effect on the carcass and, thigh, drumstick, breast, back, spleen, liver, gizzard, heart, bursa and thymus percentages. These deduce that the different inclusion levels of choline used do not interfere with muscle development and also prevent abnormal accumulation of fat or development of

fatty liver. However, the dietary supplementation of choline significantly ($P < 0.05$) influenced the dressing percentage. This result disagrees with DENG and WANG (1997), who observed that the addition of betaine did not affect dressing percentage of broiler chicks. The dietary supplementation of choline in this research showed a slight increase but did not significantly ($P > 0.05$) affect the breast percentage of broiler chickens at 49 days. Similarly, ESTEVE-GARCIA and MACK (2000) reported that the effects of betaine on breast yield were relatively small and non-significant. This agrees with WALDROP and FRITIS (2005) who reported an improvement in breast yield due to choline supplementation (1000 g choline/ton diet) at 42 days of age but not at 49 days of age in broiler chicks. On the contrary, ROSTAGNO and PACK (1996), REMUS (2001) and WALDROUP and FRITTS (2005), reported that the breast meat percentage was increased as diets containing different levels of betaine were fed. The present results are in accordance with the findings of KHOSRAVINIA et al. (2015), who reported an improvement in body weight and FCR but no change in carcass yield percentage when broilers were fed moderate energy diets supplemented with Bio choline, choline chloride and lecithin extract. Also, JAHANIAN and RAHMAN (2008) reported the effects of betaine supplementation to increase dressing and breast meat percentage but no significant effect on thigh and liver weight percentage, compared to a control diet without betaine.

In conclusion, the feed conversion ratio (FCR) of Cobb 500 broiler chickens at the starter phase (day 21) had a significant effect at 1200 ppm/kg of feed dietary supplementation of choline and dietary supplementation of choline at 800 ppm/100 kg of feed, reduced the feed intake of broiler chickens at finisher phase (day 49). Dietary supplementation of choline at 800 ppm significantly influences the carcass yield (dressing percentage and thigh) of broiler chickens.

Based on the present study, it is recommended to use 1200 ppm of choline (T2) as a feed additive in the diet of Cobb 500 broiler chickens at the starter phase, and 800 ppm of choline should be used as a feed additive in the diets of the broiler chickens at finishers phase.

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QUALITATIVE YIELD OF WHITE PEPPER EXTRACT OPTIMIZED BY MICROWAVE EXTRACTION AND MEAT QUALITY ASSESSMENT – AN ALTERNATIVE APPROACH

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Key words: microwave extraction, Piper nigrum extracts, optimization, meat quality, bioactive compounds.

Abstract

This study experiments the application of closed microwave extraction on aqueous white pepper–guided by two fixed [microwave power (300 W) and sieve size (0.40 mm)] and two variable [irradiation time (75–85 min) and solvent volume (280–300 mL)] factors in a central composite design. Extracts generated were optimized via meat quality assessment. From responses generated post-optimization, twelve solutions were proffered. Five solutions had highest desirability value of 0.604. Extraction criteria for recommended desirability require microwave power of 300 W, ground white pepper screened at 0.40 mm, irradiation time of 91.19 min and 280 mL of solvent volume (distilled water), but the other four solutions all require 280 mL of solvent volume and 91.151, 91.131, 91.241 and 91.091 min of irradiation time respectively. Gas Chromatography Mass Spectrometry (GC-MS) analysis of the recommended extract had a remarkable yield of forty-one (41) compounds. This green extraction procedure shows promise for future extractions.

Introduction

High solvent volume and extended extraction periods characterize traditional extraction processes that most times result in low quality yield.

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Microwave extraction have been reported as an efficient technique to obtain bioactive compounds from simple and complex herbal mixtures (Laroze et al. 2008, Neuchter et al. 2004). Closed and open microwave heating systems have been explored with significant yield, safe processes and better outputs (Olalere et al. 2017). Microwave extraction in controlled setup prevents thermo-chemical degradation of heat-sensitive phenolic compounds (Olalere and Gan 2020)–an advantage in the field of therapeutics, agriculture and medicine.

Plants contain natural antioxidants and are of considerable interest for the development of new medicines (Amal Belakredar et al. 2021). Bio-active components in Piper species have served therapeutic functions (Barh et al. 2013). White pepper (*Piper nigrum*) corm commonly found in Western countries is a ripe berry devoid of its outer skin (Agbor et al., 2006). Piper species are traditionally consumed to treat ailments or alternatively applied as preservative, insecticide or anti-microbial (Aziz et al. 2015, Gasparetto et al. 2017). Compositional analysis of oleoresin extracted by Olalere et al. (2018) yielded 31 bioactive compounds and approaches to increase extraction yield is desirable.

Application of meat quality assessment as an optimization tool is a new approach to increase extraction yield with less repetitive processes under environmentally friendly conditions. This procedure leverage on a rationale that the higher the antioxidants in extracts, the likelier the effectiveness of extraction. Therefore, an assessment of meat quality as it influences the quality of extract produced is based on the knowledge that oxidation of lipids post-slaughter can negatively affect the quality of fresh meat (Insani et al. 2008, Trout 2003). This study therefore uses an alternative approach to optimize the qualitative yield of white pepper extract via microwave extraction and meat quality assessment. The study aims to generate criteria for extracting white pepper; to optimize extraction outcomes using meat quality evaluations and to evaluate extraction efficiency using GC-MS (Gas Chromatography – Mass Spectrometry) analysis.

Materials and Methods

Experimental site

Microwave extraction was performed at the Laboratory of Feed Quality, Department of Animal Production and Health, Federal University of Agriculture, Abeokuta. Meat colour and thiobarbituric acid reactive substance value were measured at Laboratory of Food Science and Technology

and Laboratory of Veterinary Medicine; Federal University of Agriculture, Abeokuta. Compositional analysis (GC-MS) of extract with the best suggested desirability was performed at the Laboratory of Chemistry, Faculty of Science, University of Lagos.

Experimental design

Two numerical factors (independent variables): irradiation time (75–85 min) and solvent volume (280–300 mL) were varied alongside two fixed factors (microwave power and sieve size were held constant at 320 W and 0.40 mm respectively) to generate thirteen experimental runs. Central Composite Design (CCD) was employed for process analysis, while response surface methodology was applied for the optimization phase as described by ANDERSON and WHITCOMB (2016). Thereafter, Gas Chromatography and Mass Spectrometry analysis of the optimized extract with the best desirability was performed accordingly.

Sourcing and preparation of test materials

Dried white pepper corm was sourced from a renowned herb market and pulverized into finely defined powder using an attrition mill. Powdered sample was clarified using 0.105 mm sieve prior to storage in air – tight amber coloured vials. Hi-sense (H36MOMMI) microwave was used for extraction process, while distilled water was used as solvent.

Twenty-five (4-weeks old) broiler chickens intensively raised under deep litter management system for five (5) weeks were subjected to uniform management. Commercial diet fed is shown in Table 2. Afterwards, the birds were sacrificed and the breast muscles were extracted and weighed prior to use.

Ethical guideline. Ethical guidelines were strictly adhered to prior to slaughter following the established guidelines established by the Animal Welfare and Ethics Committee of the College of Animal Science and Livestock Production, Federal University of Agriculture, Abeokuta.

Microwave extraction procedure. Thirteen (13) experimental runs were proposed by Design Expert software (5 repetitions generated as centre point). Eight (8) grams of ground white pepper was dissolved in each run comprising 275.86–304.14 mL of distilled water. Solute was stirred until homogeneity and uniformity was attained. The of mixture was placed in an irradiation-tolerant container before placement in the microwave cavity. Pre-heating of the cavity was performed for 15 min at 100 W. Afterwards, 300 W power was set and extraction was performed following designed sug-

gestions (Table 1). Subsequently, loading and unloading of mixture and extracts from the cavity was carried out according to procedure established by previous studies (OLALERE et al. 2017 2018). Next, extracts obtained were cooled and stored in coloured vials prior to meat quality assessment.

Extract yield calculation. The percentage yield of extraction is expressed as follows:

$$\text{Extraction Yield} = \frac{volume\ of\ extracts\ [\text{ml}]}{volume\ of\ mixture\ [\text{ml}]} \cdot 100.$$

Gas chromatography mass spectrophotometry of aqueous white pepper extract. Gas Chromatography Mass Spectrophotometry analysis of extract suggested desirability was performed. Filtered extract (1 μL) was diluted using an analytical standard grade acetone extract at 1 : 10 ratio and injected into the column for components identification according to OLALERE et al. (2018). Compounds identified in relation to the peak area fragmentation fingerprints were recorded.

Statistical design

Data obtained from responses were analysed using Design Expert version 12.0.3.0 (D_x 12, 2019). Mean separation at 5% level of significance was carried out by subjecting regression coefficients to Analysis of Variance (ANOVA) to obtain coefficient of determination (R^2) for each response. Numerical optimization was carried out to ascertain the level of desirability.

Table 1

Central composite design matrix for extraction of aqueous white pepper

RUN	Irradiation time [min]	Solvent volume [mL]
1	2	3
1	90.00	304.14
2*	90.00	290.00
3	95.00	300.00
4	85.00	300.00
5*	90.00	290.00
6*	90.00	290.00
7	82.93	290.00
8*	90.00	290.00
9	85.00	280.00
10*	90.00	290.00
11	97.07	290.00

cont. table 1

1	2	3
12	95.00	280.00
13	90.00	275.89

Explanations: *****midpoint repeated five times

Table 2

Nutrient composition of commercial finisher diet fed broiler chickens

Ingredient (DM)	Composition
Energy [Kcal]	2900
Crude protein [%]	20.00
Fat/Oil [%]	6.00
Crude fibre [%]	5.00
Salt [%]	0.30
Lysine [%]	0.85
Methionine [%]	0.35
Calcium [%]	1.00
Available Phosphorus [%]	0.40

Explanations: DM – dry matter, Kcal – kilocalories, % – percentage

Data collection

Extract uptake. Fifteen (15) grams of meat from the breast was weighed out in triplicates. Fifteen (15) mL of extract was added to each replicate and soaked for 30 min. Samples were subsequently removed and reweighed after 5 min. Increase in weight of samples indicate the volume of extract absorbed, and weight change was expressed as a percentage of the initial weight of meat before soaking. Afterwards, qualitative evaluations were carried out on meat samples that contain extracts.

Determination of microwave internal temperature. An LCD digital thermometer (MEXTECH) (St-9283B) probe was inserted into the microwave (Hi-sense H36MOMMI) cavity for 5 min post-extraction, and temperature range of 39 ±2^{o}C was recorded.

Evaluation of extract quality using meat quality analysis

pH assessment. Measurement of pH of meat was carried out with an ATC pH meter (Hanna Instruments) as described by KIM et al. (2009). Measurements were repeated on d 5 and 10. Colour measurements were determined using Chroma meter model – CR-400 (Konica Minolta, Tokyo, Japan). Colour categorization was based on 2 points on each meat sample.

Refrigeration loss [%]. Refrigeration loss percentage of meat samples containing extracts was evaluated:

$$\text{Refrigeration loss } [\%] = \frac{weight\ before\ refrigeration\ -\ weight\ after\ refrigeration}{weight\ before\ refrigeration} \cdot 100$$

Refrigeration loss percentages on d 0, 5 and 10 were recorded.

Cooking loss [%]. On day-10, post refrigeration loss analysis, meat cooking loss was determined. Samples were allowed to drain, then cooked in water bath at 65°C for 30 minutes to calculate the cooking loss percentage. After cooking, the residual moisture was allowed to drain, then weighed as follows:

$$\text{cooking loss } = weight\ before\ cooking - weight\ after\ cooking$$

$$\text{cooking loss } [\%] = \frac{weight\ before\ cooking - weight\ after\ cooking}{weight\ before\ cooking} \cdot 100$$

Oxidative rancidity measurement of meat samples containing extracts. Each meat sample (5 g) containing extract was homogenized in 15 ml of distilled water. Sample homogenate (5 ml) was transferred to a test tube and the lipid oxidation was measured as thiobarbituric acid reactive substances (TBARS) (BIDLACK et al. 1973), using an absorbance standardized at 532 nm.

$$T\text{BARS mg}\frac{\text{MDA}}{\text{kg}} of\ meat = (\text{kg of meat absorbance of sample} - \text{absorbance of blank sample}) \cdot 5.88$$

Result

Quality characteristics of chicken meat incorporated with white pepper aqueous extracts

The result for optimization of white pepper extract using broiler chicken meat is presented (Table 3). Extract volume from extraction process yielded 41.08–136.96 mL, while aqueous uptake by meat was between 0.4 and 20.33%. Meat pH of soaked samples on d 0, 5 and 10 ranged between 5.61–6.33%, 6.05–6.25% and 6.43–7.19% respectively. Meat TBARS (malondialdehyde value) ranged from 0.277–0.97, and 0.157–0.397 on day (d) 5 and 10 respectively. Meat refrigeration loss were 9.97–19.83% and 18.19–33.74% respectively on d 5 and 10, while cook loss was between 17.54 and 40.23%. Meat L*, a* and b* ranged between 59.25–76.81, 5.19–11.24 and 13.12–18.75 respectively on d 5 and 61.32–72.71, 8.44–14.53 and 14.82–17.76 respectively on d 10 of storage (4°C).

Table 3

Mean values of quality indices of chicken meat incorporated with white pepper extracts

RUN	Time [min]	Sol. Vol. [mL]	Aq. Ext. [mL]	Ext. Yield %	Ext. Abs [%]	Meat pH d 0	Meat pH d 5	Meat pH d 10	TBARS d 5	TBARS d 10	Ref. Ls d 5 [%]	Ref. Ls d 10 [%]	L*5	L*10	a*5	a*10	b*5	b*10	Ck ls d 10 [%]
1	90.00	304.14	116.25	37.24	8.97	6.33	6.15	6.97	0.34	0.32	19.83	30.26	71.49	66.72	8.03	12.97	17.09	14.53	31.30
2	90.00	290.00	127.98	42.95	7.49	6.16	6.18	6.99	0.57	0.25	15.82	27.23	67.74	67.89	11.24	14.38	16.30	13.26	32.36
3	95.00	300.00	85.88	27.88	2.33	6.33	6.10	6.43	0.97	0.18	15.36	24.72	70.97	65.87	9.49	8.48	18.66	16.00	37.5
4	85.00	300.00	136.96	44.47	6.11	6.17	6.25	6.80	0.40	0.18	18.25	32.3	76.81	72.71	8.31	9.65	18.75	15.88	34.82
5	90.00	290.00	118.58	39.79	10.15	6.02	6.18	6.44	0.43	0.22	19.19	27.42	68.46	67.86	8.61	9.80	18.67	16.78	38.57
6	90.00	290.00	122.9	41.24	7.63	6.20	6.20	6.63	2.82	0.30	17.15	30.31	86.88	68.61	5.19	14.53	14.96	15.23	25.93
7	82.93	290.00	109.28	36.67	20.33	6.12	6.11	6.50	0.39	0.31	19.07	33.74	67.32	68.65	6.38	9.29	16.77	17.30	36.14
8	90.00	290.00	75.70	25.40	7.17	5.98	6.09	6.90	0.67	0.31	16.25	26.73	70.76	66.96	6.03	8.44	16.60	14.82	36.84
9	85.00	280.00	69.00	23.96	0.40	6.29	6.05	6.51	0.28	0.30	11.33	27.61	76.28	68.96	6.70	10.75	14.70	17.76	17.54
10	90.00	290.00	76.18	25.56	9.04	6.15	6.09	7.19	0.39	0.40	15.49	23.89	67.59	71.02	5.22	11.93	14.72	16.65	29.38
11	97.07	290.00	59.27	19.89	14.36	6.20	6.10	6.69	0.36	0.27	9.97	18.19	69.4	68.57	7.41	12.33	17.32	16.12	35.53
12	95.00	280.00	60.92	21.15	8.16	5.61	6.09	6.43	0.58	0.21	15.31	28.62	59.25	69.79	6.68	11.57	13.12	17.61	40.23
13	90.00	275.86	41.08	14.47	7.10	6.20	6.07	6.57	0.63	0.16	14.20	30.53	65.64	61.32	7.37	11.12	17.48	14.97	31.72

Explanations: Ext .– extract; Abs – absorbed; Vol. – volume; TBARS – thiobarbituric acid reactive substances; Ref. – refrigeration; L* – Lightness; a* – redness; b* – yellowness; Aq. – aqueous; Ck. – cook; Ls – loss

Figure 1–7 shows the trend of influence irradiation time and solvent volume exert on meat quality. Figure 1 shows the response surface and contour plots derived from extract yield and meat solvent absorption. Extract yield was reduced as irradiation time and solvent volume increased. The volume of extract absorbed increased among extracts generated with 293 and 300 mL of distilled water and 85 min of irradiation time. Figure 2 shows the response surface and contour plots of pH of meat soaked in extracts on day (d) 0, 5 and 10 of refrigeration storage. Extracts produced from 91.25 min and 287.5 mL of irradiation time and solvent volume respectively lowered meat pH on d 0, but on d 5, pH was least at 85.5 min and 280.5 mL of time and solvent combination respectively. On d 10, meat pH of 6.5 was recorded for extracts produced with 282 mL and 85.5 min of solvent volume and irradiation time respectively. Figure 3 presents the response surface and contour plots for 2-thiobarbituric acid reactive substance value of meat of broiler chickens containing aqueous extract on d 5 and 10 of refrigeration storage. The TBARs on d 5 was lowered in sample containing extract generated below or beyond 85 and 95 min of irradiation, though highest at 90.5 min and 285 mL of irradiation time and solvent volume respectively; while oxidative rancidity measured reduced as exposure to irradiation extends beyond 95 min and solvent volume lowered beyond 280 mL of distilled water.

Response surface and contour plots of refrigeration and cook loss percentages of chicken meat soaked in white pepper aqueous extract on d 5 and 10 is presented (Figure 4). Refrigeration loss [%] of meat on d 5 was minimal for meat containing extract produced between 85.5 and 94.75 min of irradiation exposure as well as 282 and 284 mL of solvent volume; though on d 10, a combination of 287 mL of solvent and 94 min of irradiation exposure along with other set criteria yielded extracts that highly lowered meat pH.

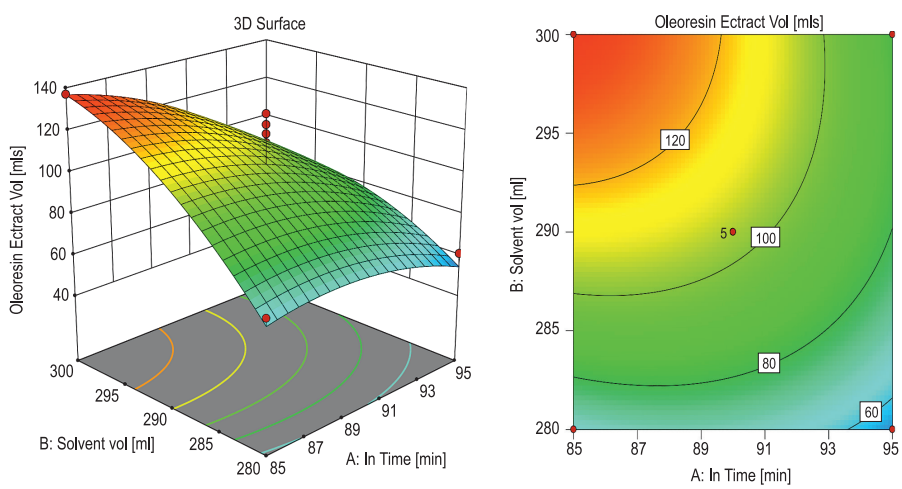

Fig. 1. Response surface and contour plots for extract yield and meat aqueous extract absorption

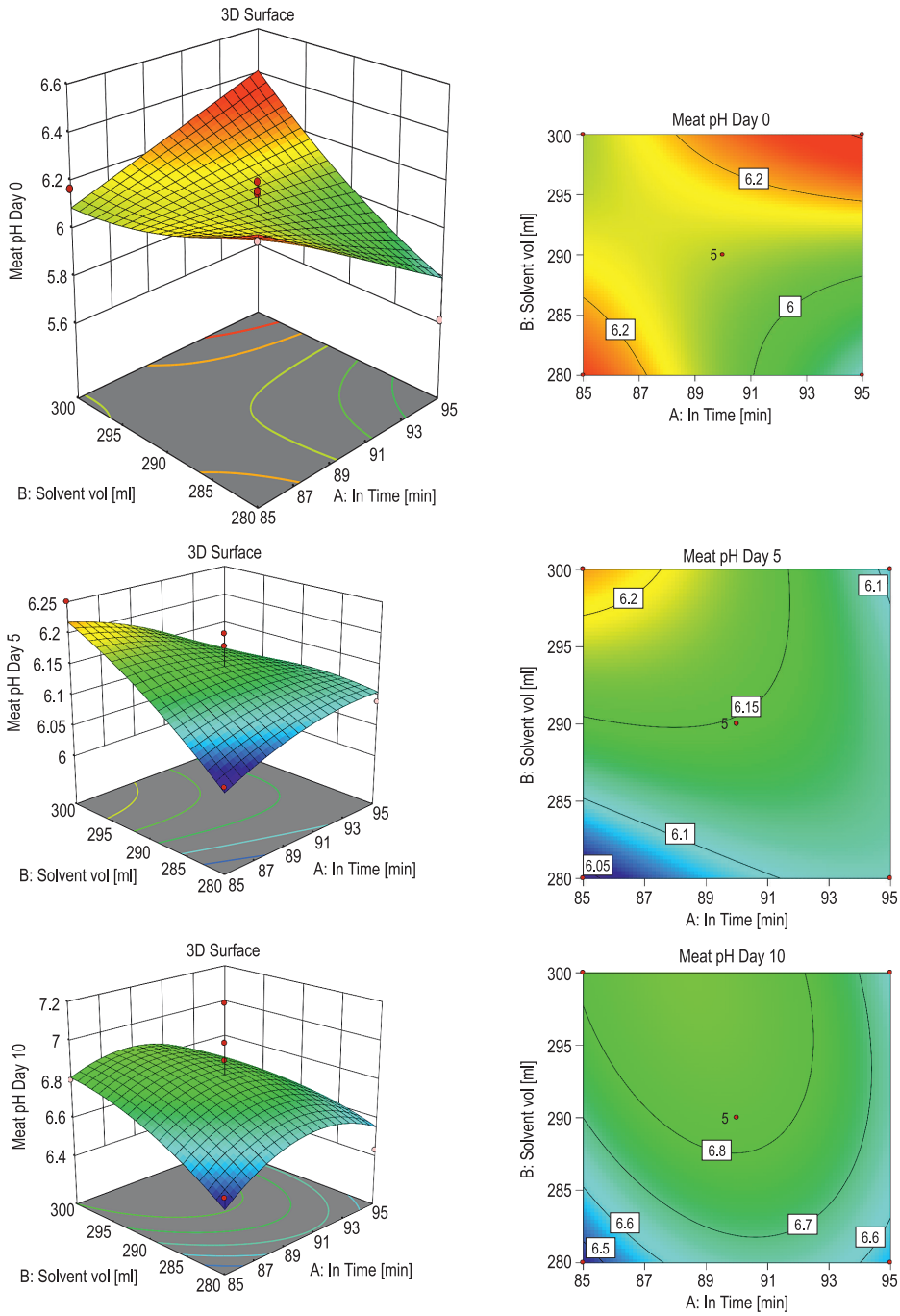

Fig. 2. Response surface and contour plots of pH of meat containing white pepper aqueous extract on d 0, 5 and 10 of storage (4°C)

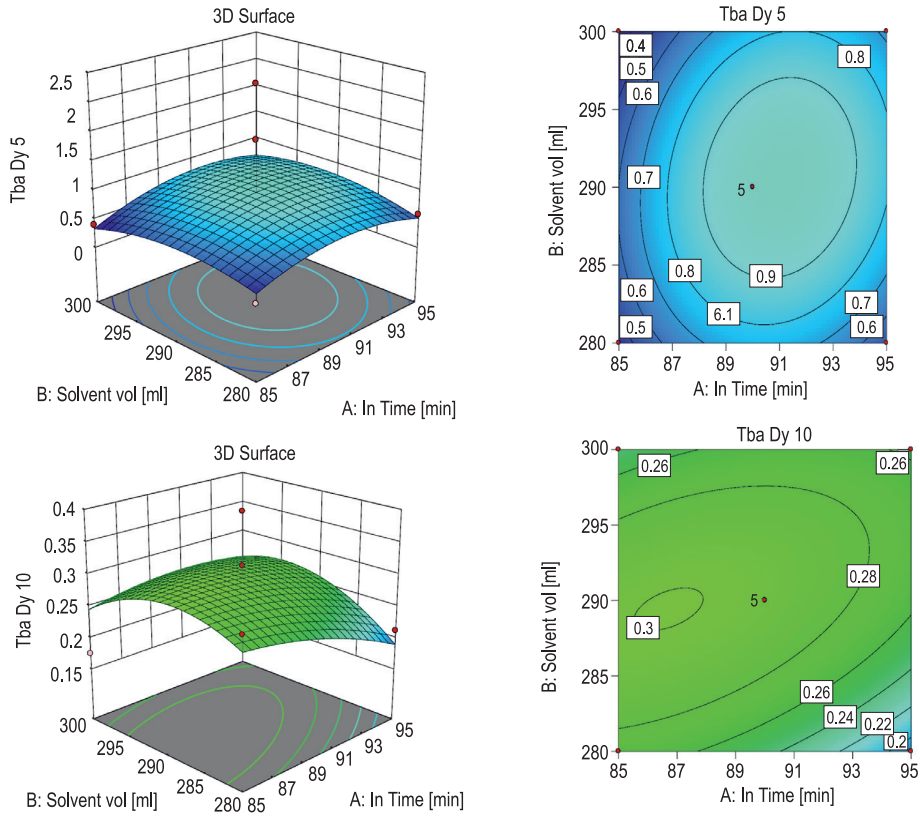

Fig. 3. Response surface and contour plots of 2-thiobarbituric acid value of meat containing extract on d 5 and 10 of refrigeration storage

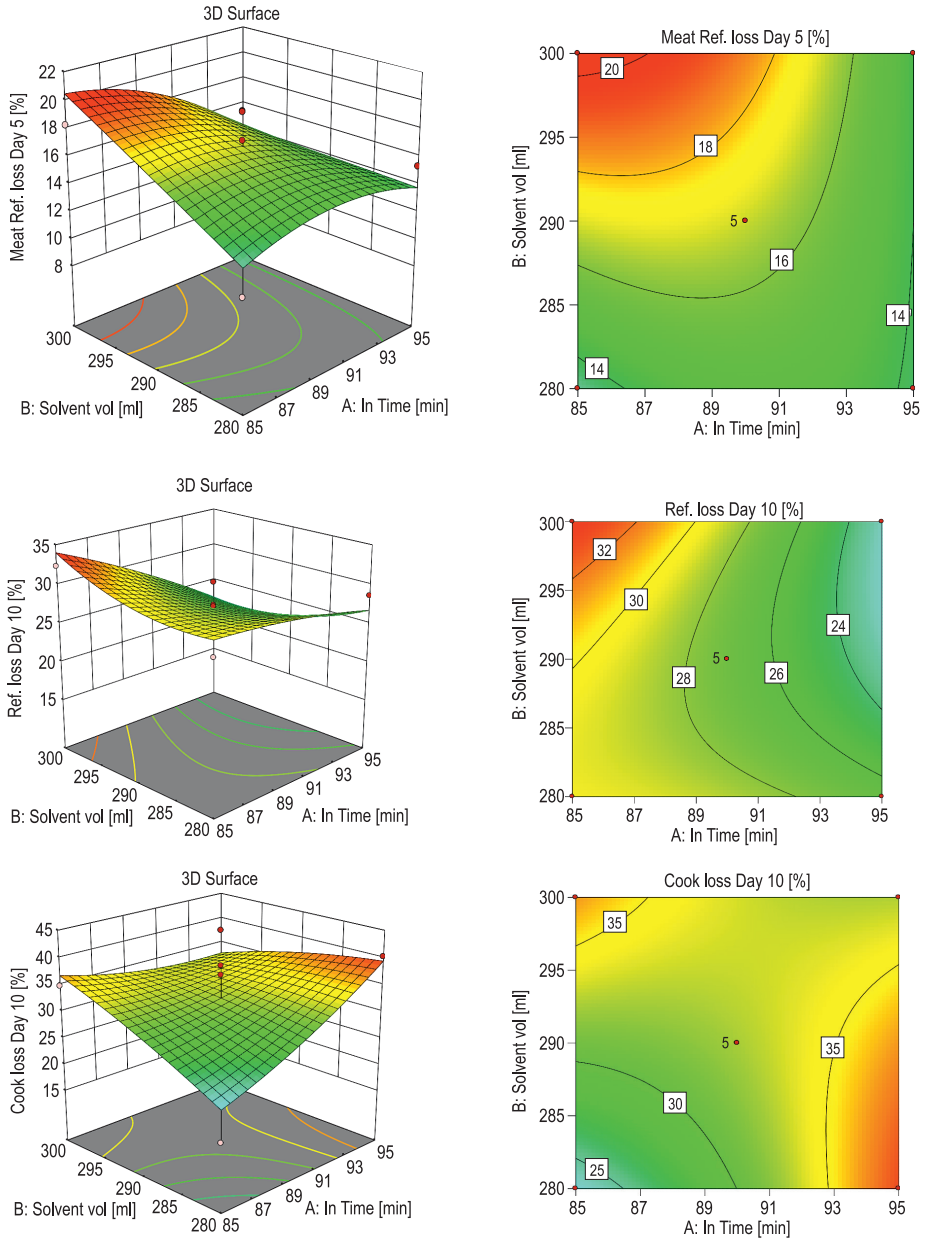

Fig. 4. Response surface and contour plots of refrigeration and cook loss of meat containing extract on d 5 and 10 of refrigeration storage

Cook loss [%] was very minimal for samples soaked in extract generated from 86.5 min and 282 mL of irradiation time and solvent volume respectively. Response surface and contour plots for Lightness (L^*) of meat containing extract on d 5 and 10 of refrigeration storage is shown (Figure 5).

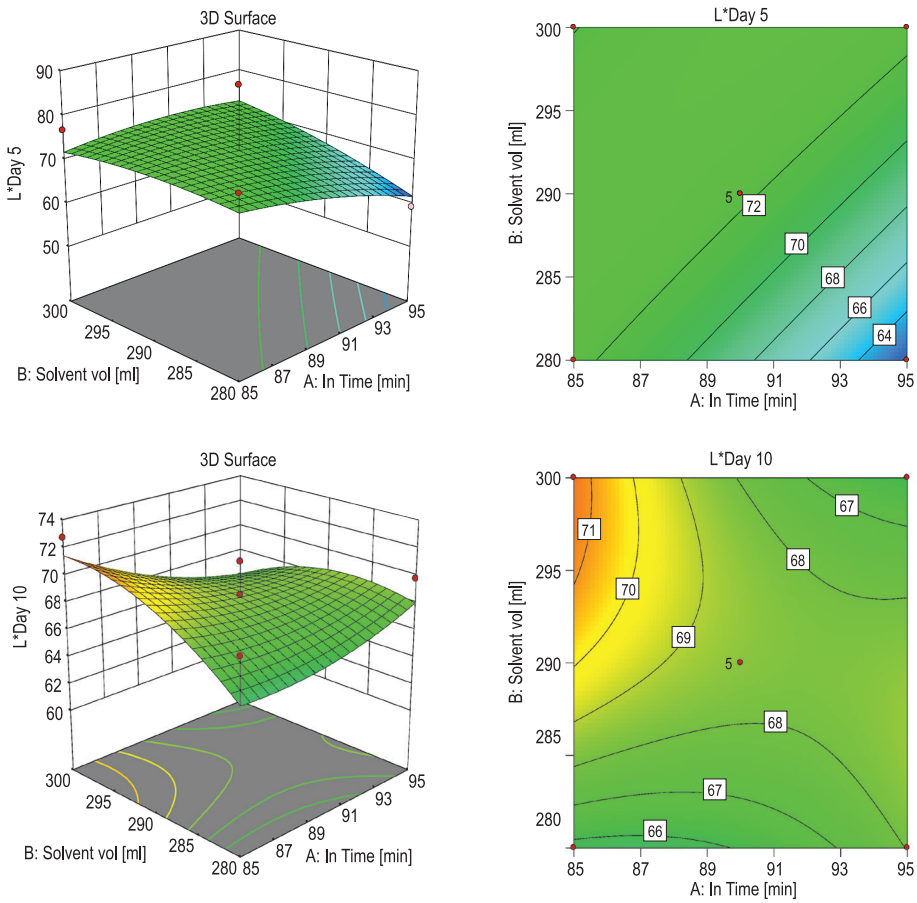

Fig. 5. Response surface and contour plots for L^* values of meat preserved with white pepper aqueous extract on d 5 and 10 of refrigeration storage

Meat L^* peaked among samples soaked in extracts produced from a combination of 90 min and 290 mL of irradiation time and solvent volume on d 5, but on d 10, samples soaked in aqueous extracts generated from 294 mL and 85.5 min of solvent volume and irradiation time respectively had the highest lightness value. Display of response surface graphs and contour plots to reveal redness (a*) value of meat after soaking in white pepper aqueous extract on d 5 and 10 is illustrated in Figure 6.

Extract generated from 94 min and 299 mL of irradiation time and solvent volume, alongside other set extraction criteria preserved meat redness, but on d 10, 91.5 and 94.5 min of irradiation time and 286 mL of solvent volume, alongside other factors yielded extracts with desirable redness, while yellowness (b^*) value of stored meat (Figure 7) was greater among samples preserved with extract generated with higher solvent volume alongside 90 min of irradiation, however, on d 10, b^* was highest among samples stored with extracts from 285.5 mL and 86.5 min of solvent volume and irradiation time respectively.

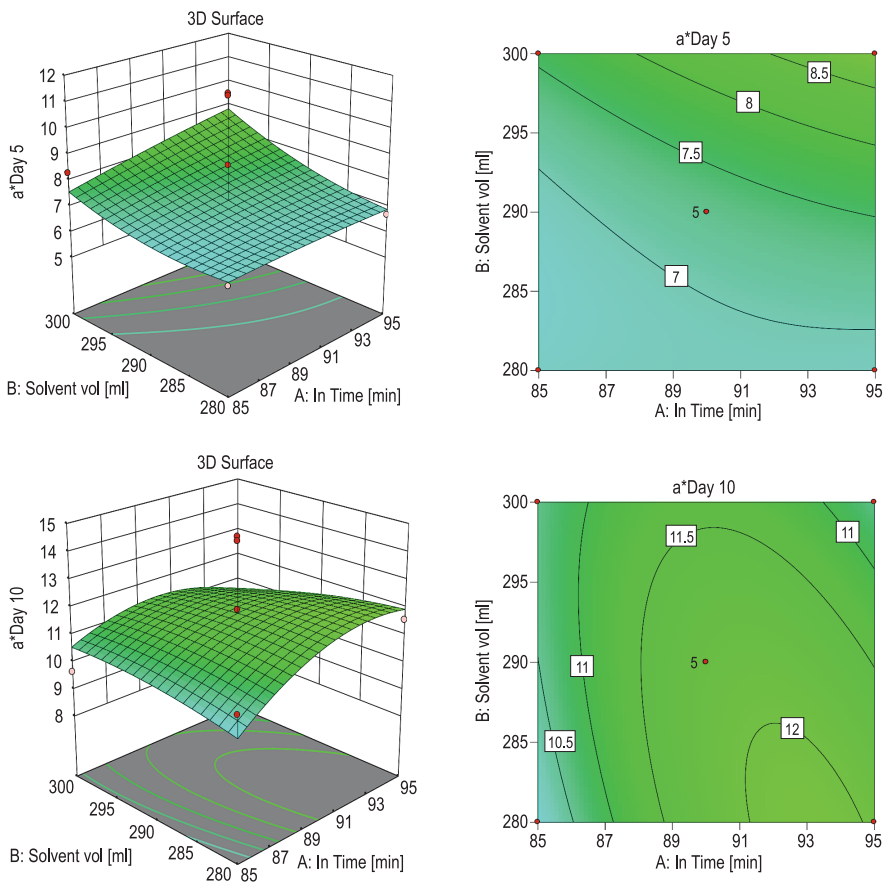

Fig. 6. Response surface and contour plots for a^* value of meat containing white pepper aqueous extract on d 5 and 10 of refrigeration storage

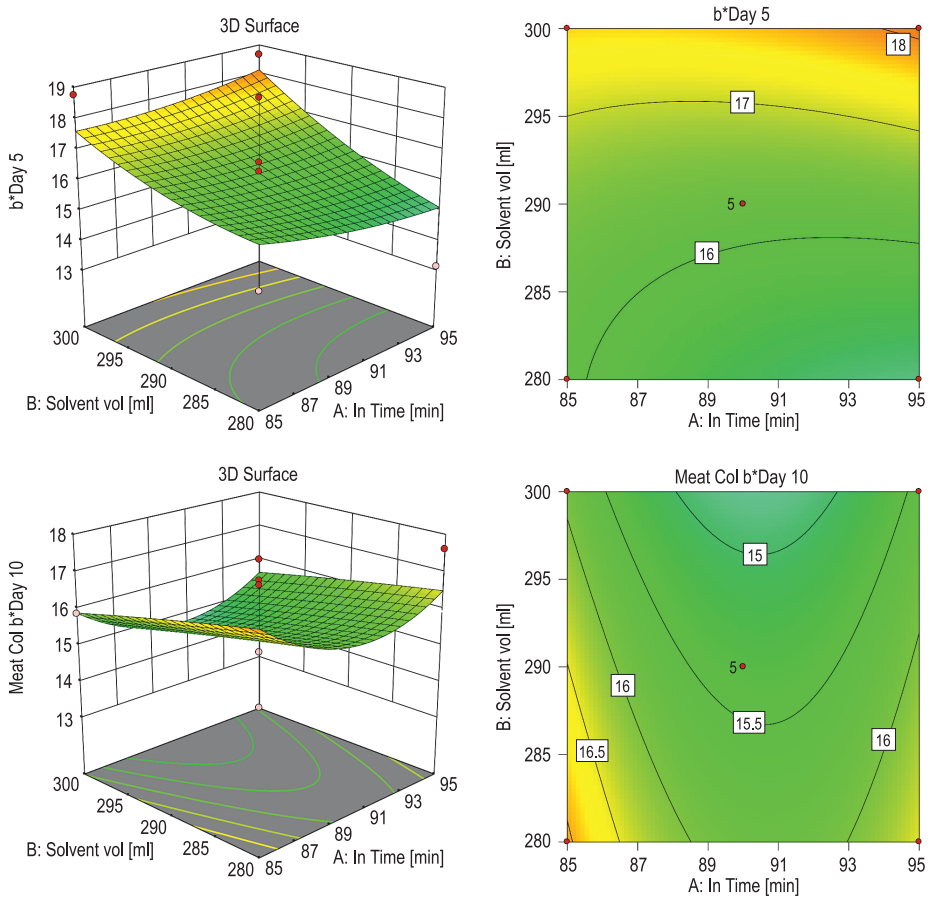

Fig. 7. Response surface and contour plots for b* value of broiler chicken meat containing white pepper aqueous extract on d 5 and 10 of refrigeration storage

Regression coefficient of responses (aqueous extract volume, meat absorption percentage, colour, loss percentage and 2-thiobarbituric acid value) as a function of the independent variables

Regression coefficient of models utilized for optimization procedure of white pepper aqueous extract is presented (Table 4). Significant ($p < 0.05$) parameters such as linear model for meat refrigeration loss percentage [%] on d 10; pH and refrigeration loss % on d 5 as well as 2FI (factor interaction) model for meat pH on d 0 were shown. Range of linear irradiation value was between -17.68 to 3.06 for aqueous extract volume and cook loss on d 10 were respectively documented.

Table 4

Regression coefficient of response parameters of white pepper aqueous extract applied as a function of independent variable

Specification	Intercept (β^0)	A – Irradiation time	B – solvent volume	AB	A^2	B^2	A^2B	AB^2	R^2
Aqueous Extract Vol	104.268	-16.2356	**24.9033***	-10.75	-8.3165	-11.1215	–	–	0.7539
p-values	–	0.0569	**0.0101***	0.3219	0.3129	0.1893	–	–	–
Aqueous Uptake	8.296	-0.557857	0.315572	-2.885	2.4145	-2.2405	–	–	0.4218
p-values	–	0.7575	0.8610	0.2788	0.2361	0.2683	–	–	–
Meat pH d 0	6.102	0.0282843	0.0459619	**0.21***	0.000875	0.053375	0.104038	-0.158284	0.6870
p-values	–	0.6336	0.4473	**0.0131***	0.9843	0.2625	0.2443	0.1010	–
Meat pH d 5	6.148	-0.0155178	**0.0403921***	-0.0475	-0.01775	-0.01525	–	–	0.6577
p-values	–	0.3631	**0.0391***	0.0733	0.3340	0.4023	–	–	–
Meat pH d 10	6.83	-0.0226624	0.106961	-0.0725	-0.1525	-0.065	–	–	0.3862
p-values	–	0.8108	0.2791	0.5915	0.1628	0.5274	–	–	–
TBARs d 5	0.9756	0.1048	0.0127651	0.067	-0.268987	-0.213737	–	–	0.1557
p-values	–	0.7235	0.9655	0.8725	0.4073	0.5062	–	–	–
TBARs d 10	0.2952	-0.0173817	0.0104914	0.023	-0.014225	-0.040225	–	–	0.2954
p-values	–	0.5416	0.7101	0.5673	0.6394	0.2088	–	–	–
Meat ref. loss Day-5	16.78	-1.47242	**1.8665***	-1.7175	-1.30625	-0.05875	–	–	0.6680
p-values	–	0.1019	**0.0485***	0.1646	0.1635	0.9461	–	–	–
Ref. loss d 10	27.116	**-3.57013***	0.0510203	-2.1475	-0.542375	1.67262	–	–	0.7392
p-values	–	**0.0072***	0.9588	0.1549	0.6118	0.1455	–	–	–
Cook loss d 10	32.616	3.06342	1.7445	-5.0025	1.322	-0.8405	–	–	0.4922
p-values	–	0.1710	0.4138	0.1216	0.5587	0.7079	–	–	–
L*d 5	72.286	-2.49105	2.56539	2.7975	-1.37175	-1.26925	–	–	0.2959
p-values	–	0.3642	0.3510	0.4662	0.6336	0.6588	–	–	–
L* d 10	68.468	-0.765392	0.933344	-1.9175	0.825375	-1.46962	–	–	0.5510
p-values	–	0.3935	0.3042	0.1513	0.3910	0.1476	–	–	–
a*d 5	7.258	0.32708	0.669173	0.3	-0.057125	0.345375	–	–	0.1613
p-values	–	0.6666	0.3883	0.7791	0.9437	0.6714	–	–	–
a*d 10	11.816	0.493651	-0.196713	-0.4975	-0.83175	-0.21425	–	–	0.1644
p-values	–	0.5837	0.8255	0.6945	0.3968	0.8228	–	–	–
b* d 5	16.25	-0.111523	1.12981	0.3725	0.183125	0.303125	–	–	0.3174
p-values	–	0.8723	0.1351	0.7055	0.8059	0.6853	–	–	–
Meat b* d 10	15.348	-0.212347	-0.514032	0.0675	0.951625	-0.028375	–	–	0.4253
p-values	–	0.6617	0.3055	0.9211	0.0980	0.9562	–	–	–

Explanations: * – significant model terms; Irr – irradiation; TBARs – 2-thiobarbituric acid value; d – day; Ref. – refrigeration; L* – lightness; a* – redness; b* – yellowness

Also, linear model for solvent volume ranged between -0.197 and 26.57 for meat L* and aqueous absorption values on d 10 of storage respectively, similarly as 2FI model for aqueous extract volume and meat pH on d 0. Meat quadratic model was not significant ($p > 0.05$) but ranged between -8.317 and 2.415 for extract volume and absorption percentage, while quadratic model for solvent volume for extract volume and refrigeration loss were -11.122 and 1.67 on d 10 of storage. R^2 values (coefficient of determination) was 0.1557 for TBARs on d 5 and 0.7392 for refrigeration loss on d 10.

Responses suggested for the optimization of *Piper nigrum* aqueous extract assessment

A total of twelve (12) solutions were proffered by RSM (Table 5), with a desirability range of 0.587–0.604 or 58.7–60.4%. To prepare extract of suggested desirability, 91.19 min of irradiation time and 280 ml of solvent volume were conditioned alongside 300 W of microwave power and *P. nigrum* powder screened at 0.40 mm.

Gas chromatography-mass spectrometry of optimized white pepper aqueous extracts

The GC-MS result for optimized white pepper aqueous extract of best desirability is reported (Table 6). A total of 41 compounds were identified. One silicon and sulphur-based compound was gotten. Stearic acid, iso-octyl phthalate and Bis (2-ethylhexyl) phthalate were present in high proportion than all other compounds, followed by 2-phenylethanol–a terpenoid. Dibutyl phthalate present was moderately abundant alongside *Phthalic acid and butyl hexyl ester. Next is palmitic acid, then* Octamethylcyclotetrasiloxane (D4) and terpenoid alkaloids such as γterpinene, Terpinen-4-OL and α-terpineol. Flavonoids such as eicodecene and octadecanoic acid were present. As shown in Figure 8, least quantity of 0.74 for p-nitrobenzaldehyde pale in area compared to highest value of 24.65 for isoctyl phthalate and bis (2-ethylhexyl) phthalate.

Table 5

Solutions for numerical optimization of responses using white and black pepper aqueous extract on broiler chicken meat

Number	Time	Solvent vol.	Aqueous extract vol.	Ext. Abs.	Meat pH D 0	Meat pH D 5	Meat pH D 10	TBARS D 5	TBARS D 10	Meat ref. loss D 5	Ref. loss D 10	Cook loss D 10	L* D 5	L* D 10	a*- D 5	a* D-10	b* D 5	Meat Col b* D 10	Desirability
1	91.188	280.000	66.470	6.429	5.995	6.099	6.661	0.743	0.234	14.839	28.369	32.022	67.117	66.385	6.937	11.987	15.319	15.821	0.604
2	91.151	280.000	66.539	6.404	5.997	6.099	6.661	0.744	0.234	14.842	28.381	31.958	67.161	66.374	6.937	11.983	15.322	15.820	0.604
3	91.139	280.000	66.561	6.396	5.998	6.099	6.661	0.744	0.235	14.843	28.385	31.938	67.175	66.370	6.937	11.981	15.323	15.819	0.604
4	91.241	280.000	66.370	6.466	5.993	6.099	6.661	0.742	0.234	14.835	28.351	32.114	67.054	66.402	6.937	11.993	15.314	15.823	0.604
5	91.091	280.000	66.650	6.363	6.001	6.098	6.662	0.745	0.235	14.846	28.401	31.854	67.232	66.356	6.937	11.975	15.326	15.818	0.604
6	90.960	280.000	66.883	6.276	6.007	6.098	6.662	0.746	0.236	14.854	28.444	31.629	67.385	66.317	6.937	11.958	15.337	15.815	0.603
7	91.450	280.000	65.952	6.618	5.982	6.100	6.660	0.737	0.232	14.816	28.279	32.482	66.802	66.469	6.937	12.016	15.298	15.833	0.603
8	91.507	280.000	65.836	6.660	5.979	6.100	6.659	0.736	0.231	14.810	28.260	32.582	66.733	66.487	6.937	12.022	15.294	15.836	0.603
9	90.771	280.000	67.200	6.156	6.017	6.097	6.662	0.749	0.238	14.861	28.505	31.306	67.604	66.262	6.937	11.931	15.353	15.813	0.603
10	91.611	280.000	65.613	6.741	5.973	6.101	6.658	0.733	0.230	14.798	28.223	32.768	66.605	66.522	6.937	12.032	15.286	15.842	0.603
11	91.838	280.000	65.104	6.921	5.962	6.102	6.656	0.727	0.228	14.768	28.141	33.174	66.322	66.600	6.936	12.050	15.270	15.859	0.600
12	92.660	280.000	62.971	7.661	5.919	6.104	6.641	0.693	0.219	14.615	27.827	34.696	65.250	66.912	6.932	12.090	15.218	15.954	0.587

Explanations: Ext. – extract; Irr – irradiation; TBARS – 2-thiobarbituric acid reactive substances; Day – D; Ref. – refrigeration; Ab – absorption; L* –- lightness; a* – redness; b*– yellowness; Vol. – volume; Col – colour

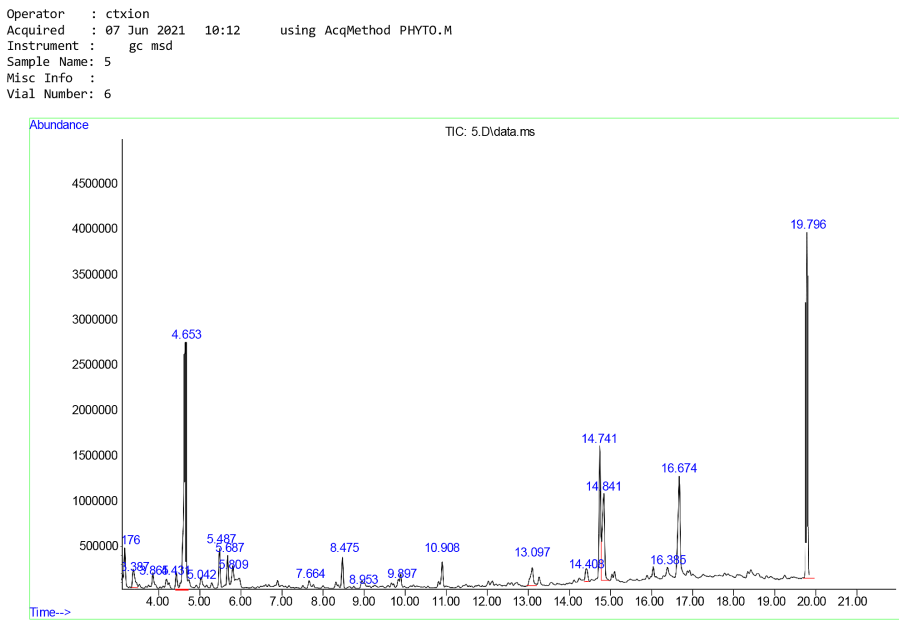

Fig. 8. Spectrometry of white pepper exctact (suggested desirability)

Table 6

Gas chromatography mass spectrometry of components of white pepper aqueous extract

Component	Molecular formula	Retention time [min]	Area [%]
1	2	3	4
Octamethylcyclotetrasiloxane (D4)	$C_8H_{24}O_4Si_4$	3.176	2.57
Hemellitol	C_9H_{12}	3.387	2.09
Pseudocumene	C_9H_{12}	3.387	2.09
Mesitylene	C_9H_{12}	3.387	2.09
3-Carene or limonene	$C_{10}H_{16}$	3.865	1.42
(E)-ß-ocimene	$C_{10}H_{16}$	3.865	1.42
Hendecane	$C_{11}H_{24}$	4.431	1.17
2-phenylethanol	$C_8H_{10}O$	4.653	21.60
α-terpineol	$C_{10}H_{18}O$	5.042	1.07
3-Ethyl-5-methoxy-1,3,4-oxadiazol-2 (3H)-one	$C_5H_8N_2O_3$	5.042	1.07
α, α-Dimethylcyclopentanemethanol	$C_8H_{16}O$	5.042	1.07
γ-terpinene	$C_{10}H_{18}O$	5.487	2.46

cont. table 6

1	2	3	4
Terpinen 4 OL	$C_{10}H_{18}O$	5.487	2.46
α-terpineol	$C_{10}H_{18}O$	5.687	2.25
(L)-alpha-Terpineol	$C_{10}H_{18}O$	5.687	2.25
a-Terpineol acetate	$C_{12}H_{20}O_2$	5.687	2.25
Dodecane	$C_{12}H_{26}$	5.809	1.52
N-Tridecane	$C_{13}H_{28}$	5.809	1.52
Heptdecane	$C_{17}H_{36}$	5.809	1.52
p-nitrobenzaldehyde	$C_7H_5NO_3$	7.664	0.74
n-tetradecane	$C_{14}H_{30}$	8.475	2.09
n-pentadecane	$C_{15}H_{32}$	8.475	2.09
p-(methoxymethyl)phenol	$C_8H_{10}O$	8.953	0.89
4-(2-hydroxyethyl) phenol	$C_8H_{10}O$	8.953	0.89
2,4-di-tert-butylphenol	$C_{14}H_{22}O$	9.879	0.81
2,5-bis(1,1-dimethylethyl) phenol	$C_{14}H_{22}O$	9.879	0.81
Cetane	$C_{16}H_{34}$	10.908	1.77
Hexacosane	$C_{26}H_{54}$	10.908	1.77
Octadecane	$C_{18}H_{36}$	13.097	2.46
Nonadecane	$C_{19}H_{40}$	13.097	2.46
Methyl palmitate	$C_{17}H_{34}O_2$	14.408	1.40
Dibutyl phthalate	$C_{16}H_{22}O_4$	14.741	9.42
Phthalic acid, butyl hexyl ester	$C_{18}H_{26}O_4$	14.741	9.42
Dibutyl phthalate	$C_{16}H_{22}O_4$	14.741	9.42
n-Hexadecanoic acid (palmitic acid)	$C_{16}H_{22}O_2$	14.841	8.13
Cyclopentadecane	$C_{16}H_{32}O_2$	16.358	1.12
1-Octadecene	$C_{15}H_{30}$	16.358	1.12
Cycloeicosane	$C_{18}H_{36}$	16.358	1.12
Octadecanoic acid (stearic acid)	$C_{20}H_{40}$	16.674	10.50
Isooctyl phthalate	$C_{18}H_{36}O_2$	19.796	24.65
Bis (2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	19.796	24.65

Discussion

From the response surface and contour plots results, it could be deduced that extract yield increases as irradiation time and solvent volume increases. This agrees with TUSHAR et al. (2017), who observed increase in the yield of oil as contact time and solvent volume increased; possibly due to increased movement or kinetics among particles accompanied by refluxation within the microwave cavity. Also, the volume of extract absorbed by meat increased as irradiation time and solvent volume increased since microwave heating at the designed conditions generally limit thermal decomposition of soluble compounds (VENTURA et al. 2017), thus increasing the availability of solute. The data obtained for meat pH for all days of refrigeration storage showed that the application of higher irradiation time and solvent volume resulted in production of extract of high acidity but the potency was short-lived compared with least pH values of 6.05 and 6.50 on d 5 and 10 generated using 85.5 min. In a study conducted by ISMAIL-SUHAIMY (2021), it was observed that increase in extraction time and microwave power caused a decrease in flavonoid yield; implying lower extraction time favors extraction of bioactive and thermal-stable compounds. Notably, those values were obtained using almost the same solvent volume (281 and 282 mL respectively) as this study, though lower than the 287.5 mL required to generate extracts that best-lowered meat pH on d 0. The same trend was observed for meat TBARs value on d 5, refrigeration loss, cooking loss and meat lightness value. These parameters were best minimized at 85.5 min of irradiation time, while refrigeration loss was best-minimized for samples containing extracts generated from 86.5 min on d 5 of storage. On the contrary, meat L^* was increased at 85.5 min of extraction. From the studies of CHEN (2015) and HABEEBULLAH et al. (2020), selectivity of extraction, biological strategies present and increased bioactive components obtained provides a wide range of active principles that minimizes the extent of spoilage. Meat a^* and b^* values were higher after soaking in extracts generated from higher extraction time (92.5–95 min) and solvent volume, yet, it was observed that as days of storage progressed, lower irradiation time and solvent volume yielded higher a^* and b^* values.

From the regression analysis table, increased extract volume correlates to increased exposure of absorbing surface to irradiation. Meat pH was significantly affected on d 10 possibly from the combination of irradiation time and solvent volume at low levels. As exposure to irradiation and extraction time extend, an associated risk of degeneration of thermolabile constituents exists (AL-HARAHSHEH and KINGMAN 2004). Therefore,

beyond threshold, extract obtained will be of low quality. This supports the report published by OLALERE et al. (2018), whose study reveal oleoresin harvested decreased beyond 120 min of irradiation exposure. Similarly, meat quality on d 10 reveal TBARs was minimal among samples containing extracts prepared from irradiation time beyond 95 min and solvent volume below 280 mL. Thermal and chemical degradation by hydrolysis, transesterification, or oxidation products controlled by rapid heating is induced by microwaves subjected to limited water content (CHAN et al. 2011, FERHAT et al. 2006, SOZMEN et al. 2012). SHASHIKANT and MAYUR (2019) affirm this by stating that the moisture/water present in heated matrix can strongly influence microwave absorption as it supports compound extraction by modifying the polarity of solvent or water applied.

Result obtained reveal irradiation time and solvent volume significantly affected meat pH on d 0 of refrigeration. White pepper extract generated using 286 mL of distilled water and 92 min of irradiation produced extracts that resulted in lowered meat pH. Extraction conditions highly favour increased acidity, corresponding to lowered pH in meat. More solvent volume effectively increased meat pH on d 5 of refrigeration. KLONT (2005) and MARTINS et al. (2018) explained that post mortem metabolism (glycolysis) and glycogen conversion into lactic acid yields highest quality products that tend to fall within a pH range of 5.7 and 6.0. Both the rate and extent of reduction of meat pH post-mortem influences meat quality characteristics. Increase in solvent volume reduces acidity levels of white pepper aqueous extract that subsequently affect meat pH value. BHATTARAI et al. (2013) reported that concentration dependence of molar volumes appears to be negligible over the entire concentration range if the molar volume remains constant. The pH of meat influences its water holding capacity (WHC)–a quality parameter closely related to product yield and quality. If lower pH values result from lower solvent volume, then the posit above indicate lower solvent volume should translate into lower refrigeration loss in meat. In this study, refrigeration loss decreased as the irradiation time increased. OSMIĆ et al. (2019) reported that excessive time and temperature of extraction negatively influence yield of total phenolics, flavonoids and anti-oxidant compounds of sage extracts. This implies that increased irradiation time likely had negative impact on the yield of bioactive substances from *P. nigrum*. RAMAN and GAIKAR (2002) affirmed that high microwave powers of 300 and 450 W increased solvent loss by 16–20%.

Conclusion

From this study, criteria to optimize extraction of functional compounds in white pepper require eight grams of white pepper; 300 W of microwave power; sieve size of 0.40 mm; 91.188 min of irradiation time and 280 mL of solvent volume. Though a total of twelve solutions were suggested, a desirability value of 0.604 was recommended for five solutions, but the GC-MS analysis of the best suggested extract reveal forty-one compounds were present–a remarkable improvement compared to outcomes of microwave extraction reported. Bio-compounds such as 2-phenylethanol, bis (2-ethylhexyl) phthalate and iso-octyl phthalate are compounds with the highest amounts extracted as seen from the GC-MS analysis carried out.

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FERRONICKEL MINING POLLUTION: HEAVY METAL ACCUMULATION, OXIDATIVE STRESS, AND BIOINDICATOR POTENTIAL OF *HELIX POMATIA* (L.) SNAILS IN DRENAS, KOSOVO

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Key words: heavy metals, pollution, oxidative stress, hepatopancreas, snail.

Abstract

Heavy metal contamination from mining activities poses significant environmental and public health risks. This study investigates the impact of the Ferronikel mine in Drenas, Kosovo, on soil heavy metal concentrations (Pb, Zn, Ni) and their subsequent bioaccumulation in *Helix pomatia* (L.) snails. 120 soil and 120 snail samples were collected at 1 km, 2 km, and 5 km radial distances from the mine, and heavy metal concentrations were measured using atomic absorption spectrometry. Oxidative stress biomarkers (protein carbonylation, malondialdehyde, and total protein) were analyzed in snail hepatopancreas tissue. Results revealed elevated heavy metal concentrations in soil and snail shells near the mine, exceeding permissible limits for Ni. Oxidative stress parameters were significantly increased in snails from contaminated sites, suggesting a direct link between heavy metal exposure and physiological damage. These findings highlight the potential of *H. pomatia* (L.) as a bioindicator for heavy metal pollution and emphasize the need for stringent environmental monitoring and mitigation strategies in mining areas to safeguard public health.

The results of this research show that there is a high concentration of these metals in the soil in polluted areas. Therefore, they bioaccumulate in the snail shell and cause oxidative stress in the hepatocytes of the tissue snail *H. pomatia* (L).

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Introduction

Heavy metals are gaining a lot of attention as environmental contaminants due to their capacity to infiltrate the food chain through polluted soil, their bioaccumulation in plants and animals, and their transfer to humans, which is currently being researched in ecotoxicological issues. Human activities such as mining, traffic, intensive agriculture, and others can cause air pollution by releasing particles in the soil or dust. This is especially true when the weather is dry and windy (OLAINKA et al. 2011), additionally, it also affects the plant and animal species that make up the food cycle of the ecosystem (JOLLY et al. 2013).

Their environmental quality is inextricably linked to the quality of agricultural goods, which in turn has an impact on people's health. As a result, soil and water-related environmental challenges are crucial for environmental contamination from heavy metals (SUN et al. 2019). The most important environmental issue is heavy metal soil contamination, which greatly impacts people's health including neurological, cardiovascular, and nephrological (SHAKERI et al. 2009).

Industrial sources of heavy metal include operations that process metal in refineries, burning coal in power plants, burn oil, have nuclear power plants, use high-tension lines, process plastics, textiles, and microelectronics, preserve wood, and process paper (BRADL 2002).

Urban and agricultural soils may become contaminated with heavy metals as a result of mining, manufacturing, and the usage of synthetic items (such as pesticides, paints, batteries, industrial waste, and the application of residential or industrial sewage to the ground).

Bioaccumulation is the buildup of absorbed chemicals in an organism over time, whereas the biomagnification is the increase in the concentration of these chemicals in each organism up the food chain (CARSON 2023).

Old landfills (especially those that accepted industrial wastes), old orchards that used insecticides with arsenic as an active ingredient, fields that had previous applications of wastewater or municipal sludge, areas in or around mining waste piles and tailings, industrial areas where chemicals may have been dumped on the ground, or areas downwind from industrial sites are examples of places where potentially contaminated soils may be found (AUBUM 2000).

Snail meat has a long history of being valued as a premium meal. Snail meat has a high protein level and a comparatively low lipid content used for food by humans and carnivores and this is a link in the food chain through which heavy metals are translocated from one link to another (MOONEY et al. 2002).

Researchers have traditionally used snails to analyze the buildup of pollutants. The assessment of internal concentrations of heavy metal contamination following a predetermined exposure period of heavy metal determines the bioaccumulation of contaminants, such as metals, and permits the possibility of determining the snails' capacity for accumulation, their bioavailability, and the intensity of the transfer of contaminants from the environment, food, and/or soil (GIMBERT et al. 2006).

In terms of snail anatomy the foot and the viscera are two important components to take into account. The kidney, hepatopancreas, heart, and a portion of the genital system that extends into the foot are among the viscera, which are the organs of the snail shell. Essentially, the foot is made up of the nervous system and the first section of the digestive tract. Pollutant concentrations in the hepatopancreas and kidney increase proportionally with exposure, reflecting both the pollutants' bioavailability to the organism and their levels in the environment (COEURDASIER et al. 2002). Snail organisms are also chosen as sentinels due to their limited toxic response or reduced ability to control their tissue levels. Studying the impact of metals and other pollutants on organism physiology contributes to the development of many toxic studies which can be used as an environmental evaluation tool (BROUDI et al. 2020). Within bioindicator species, like the *H. pomatia* (L.) snail, the accumulation of heavy metals can differ across organs, with those exhibiting high metabolic rates, such as the hepatopancreas and digestive tract, being particularly prone to elevated concentrations (DALLINGER 1993, MENTA and PARISI 2001).

Excessive (Pb) in plants affects normal metabolic pathways by interfering with particular cellular enzymes and may also prevent plants from photosynthesizing. In general, high quantities of heavy metals can cause oxidative stress, damage to DNA, and disruptions to the metabolic processes (JOSHUA et al. 2015). Generally, based on the research MOHAMMADEIN et al. (2013). The ecotoxicological approach described in the current study may have relevance to the ecological impact of several pollutants on the ecosystem and human health. Results obtained from bioaccumulation and histological responses of this common snail can give a useful indication in monitoring soil pollution by heavy metals.

The territory of the municipality of Drenas lies in the central part of Kosovo, in the valley of Drenica, 32 km from Pristina. Its territory lies between the Plain of Kosovo and that of Dukagjin and is a connecting bridge between these two regions. The municipality of Drenas has an area of 275.63 km2 with an altitude of: 575 m, the lowest point, and 1072 m, the highest point. The slope of the terrain is 10–35%. The latitude where the municipality lies is 42°32", longitude latitude is 20°64". The territory of

Drenas municipality is not uniform; it is generally composed of flat, hilly and mountainous areas. The concentrations of heavy defects according to a project financed by UNDP for the monitoring of PM10 and the determination of heavy defects in the area of Drenas, exceedances of the allowed values were recorded only for the metal Nickel (Ni). Within this project, the concentration of heavy metals in PM10 has been evaluated, for the metals Arsenic (As), Cadmium (Cd), Chromium (Chr), Copper (Cu), Mercury (Hg), Nickel (Ni), Lead (Pb), Zinc (Zn) and Iron (Fe).

According to the results from the analysis of the filters, exceedances of the allowed values (20 μg/m^3) were recorded only for the metal Nickel (Ni), in 12 cases, mainly during October, November, and December 2015 in all four sampling locations (Çikatovo and old, Çikatovo e Re, Gllobar and Lagja e Feronikeli) (UNDP/IHMK. 2015).

Materials and Methods

Applied methods

Soil samples and snail *H. pomatia* (L.) were used as material for researching the impact of heavy metal pollution in soil and their effect on oxidative stress in hepatopancreas at the locality of the Municipality of Drenas in Kosovo.

Samples of soil (120), and 120 snails were collected according to radius circles 1 km, 2 km, and 5 km from the point of contamination "Ferronikel" area. Concentric circles were divided into 4 geographical areas northwest, northeast, southeast, and southwest. Samples were collected in natural soils at a depth of 0–15 cm, and an average sample was prepared from 10 separate samples. Snails are also collected in natural habitats around the Ferronikeli mine. In addition, 30 soil samples and 10 snails were collected in the unpolluted locality Brezne – Opoja (served as a control group). Samples were collected in the period summer–autumn 2023. Statistical analysis of the data was conducted using Minitab software, employing Tukey–Kramer post-hoc tests and ANOVA to assess statistical significance. Also, the schematic figures of points with coordinates are made from the ARCmap software program.

Soil samples were collected within a 1 km radius circle centered at coordinates N 42.6821537°, E 21.0884580° and S 42.673995°, E 21.094527°. Additional samples were collected at coordinates N 42.6910814°, E 21.0972603° and N 42.6590480°, E 21.0857567° within a 2 km radius. For the 5 km radius, samples were collected at N 42.720892°, E 21.085643°, divided across four geographical areas.

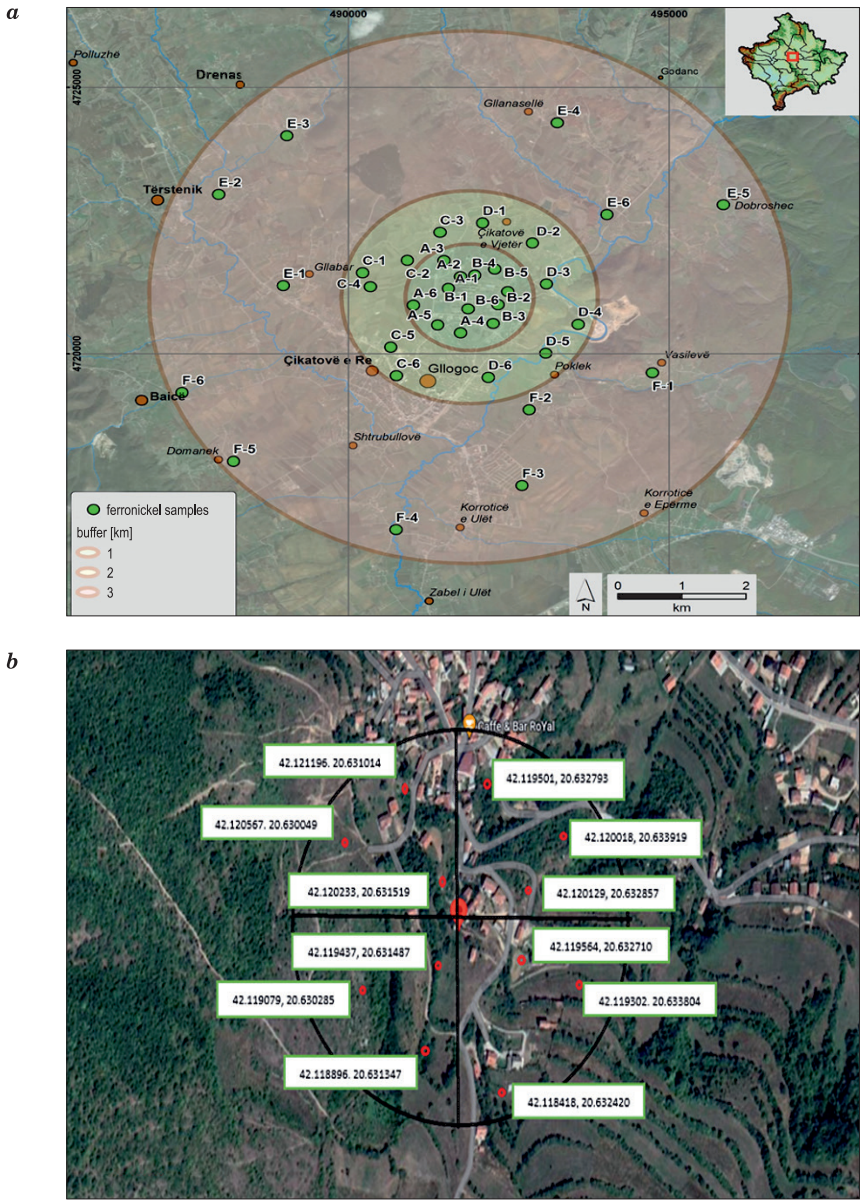

Fig. 1. Schematic representation of the sample collection points at the polluted locality Drenas a) and unpolluted site Brezne (Opoja region) b) in Kosovo

Explanations: Figure 1*a*, points: A1–A3 – Ibër Lepenc Street, northeast; A4–A6 – Mehmet Grainca Street, northwest; B1–B3 – Abedin Nika Street southeast; B4–B6 – Mehmet Grainca Street, southwest in 1 km; C1–C3 – Rrustem Grainca Street, northeast; C4–C6 – Halil Bajraktari street, northwest; D1–D3 – Ferronikel neighborhood; southeast; D4–D6 – Bajram Bajraktari Street, southwest in 2 km; E1–E3 – Istref Hoti Street northeast; E4–E6 Iber Lepenc Street; northwest; F1–F3 – Poklek; Vasileve; Korroticë e epërme; F4–F6- Korroticë e ulët, Domanek, Baincë, southwest in 5 km

Source: own study, in these two areas based on the ARCmap software program and Google Maps

This method involves from three selected points in a geographical area of radius circles are made by 10 drilling of the soil which is then mixed (*Soil quality*… DIN ISO11466. 1995). These soil samples were brought to the laboratory, ground in a soil mill and placed in glass cups and dried in a thermostat at 105°C for 48 h in order to remove moisture. They were then weighed 0.3 g after dehydration and treated with 69% nitric acid (HNO_3) and hydrochloric acid (HCl). (Merck Millipore) reagents concentrated in a 2:6 ratio in teflon columns and digested in the analyticyena TOP wave microwave at 200 C for 45 min. The contents were filtered and placed in normal 50 ml glasses in distilled H_2O. Merck Millipore ICP multi-element standard solution 111355 for metals: Lead (Pb), Zinc (Zn), and Nickel (Ni) are applied for analysis in two flame types of absorbers Thermo and Contra AAA.

In addition, snail samples were collected at the same time and place, according to the selection where previous individuals were used. The shell samples were separated from the body of snail and were washed in distilled H_2O, dried in a thermostat at 105°C for 24–48 h, then ground with a Philips kitchen mixer and 0.5 g of the sample was treated with Merck Millipore reagents; nitric acid 69% ultra-pure (HNO_3) in report 1:3 Lachner hydrogen peroxide (H_2O_2) 30% and digested in microwave at 200°C for 45 min. The contents were filtered and placed in normal glasses of 50 mL, normalized with distilled H_2O, and the metals Pb, Zn, and Ni were read in flame absorber type Analyticjena Contra AAA.

The bioaccumulation coefficient is calculated using the standard formula:

$$\mathrm{BCF} = \frac{C_{pp}}{C_s}$$

where:
C_{pp} – metal concentration in plant or animal tissue [mg/kg dry weight]
Cs – concentration in soil [mg/kg in dry weight].

Applied methods for oxidative stress

Hepatopancreatic samples for oxidative stress parameters in the vineyard snail *H. pomatia* (L.) were treated according to standard methods protocol. Thus, 36 samples of live snails per km of the locality, their shells were removed and used for the measurement of heavy metals together with soil and heath (plant) samples, then the hepatopancreas were collected from these individuals, which was then weighed and homogenized in phosphate buffer at a ratio of 9:1 mL that is (9 mL of phosphate buffer

per 100 g of living tissue) and were placed in 1.5 mL eppendorf tubes and stored in a refrigerator at -80°C. The samples were centrifuged at 3600 revolutions per minute (RPM) for 15 minutes and from the supernatant 1.5 mL of the contents were pipetted and placed in normal test tubes to which the aforementioned reagents were added.

For the measurement of lipid peroxidation, namely malondialdehyde (MDA) in the hepatopancreas, the TBARS method was applied, according to which 0.75% thiobarbituric acid (TBA), then 30% trichloracetic acid (TCA) and 5M HCl are first prepared. The contents are added to the test tubes as follows: (TBA 1.5 mL + TCA 1 mL + HCl 0.2 μL + sample 250 μL). The contents are placed in a water bath and kept at 95°C for 45 min. After this time the contents turn purple. 1 mL of the contents are pipetted and placed in the cuvette of the spectrophotometer and the absorbances at 360 and 450 nm are read. The obtained values of the samples are recorded in the database of the experiment (SYTAR et al. 2021).

To measure the carbonylation of proteins in the hepatopancreas, the 2.4 dinitrophenylhydrazine method was applied, where according to this method, the reagents are prepared: 17.20 g of 2.4 dinitrophenylhydrazine is dissolved in 41 mL of 2.5M HCl. Then 1 mL of sample is pipetted into the test tube and 4 mL of 10 mmol dinitrophenylhydrazine (DNPH) is added to it at room temperature for 1 hour so that the acid reacts with the contents and binds the carbonyl groups. After 1 hour, 5 mL of TCA 20% are added to the samples, they are centrifuged and vortexed, 6 mL of ethanol and ethyl acetate are added in a 1:1 mL ratio, they are centrifuged at 6000 RPM for 5 min. The supernatant is carefully discarded and the bottom is retained. The samples are vortexed every 15 min until the supernatant is dissolved. The supernatant is washed three times, vortexed and centrifuged in 6 mL ethanolethyl acetate. Then the bottom is dissolved in 1 mL of 6M guanidine hydrochloride in a water bath at 37°C for 10 min. The absorbances are then read and a blank acid test is performed at 360 nm in a spectrophotometer. The obtained values are recorded in the database of the experiment (STEFEK 1993).

While for the measurement of total proteins in the hepatopancreas of the snail, Lovri's method was applied. According to this method, the preparation of BSA standard 1mg/mL is done first.

Standard BSA is prepared by adding the following solutions:

a) 2% Na_2CO_3 in 0.1 N NaOH is pipetted into a 50 mL eluizer plate;
b) 1% NaK tartrate in H_2O 0.5 mL;
c) 0.5% $CuSO_4$. 5 H_2O in H_2O 0.5 mL;
d) reagent I: 48 mL of A, 1 mL of B, 1 mL of C;
e) reagent II– 50 μL Folin-Phenol [2 N].

The total protein measurement procedure is done in such a way that BSA is placed in 0 – 5 – 10 – 20 μL concentrations in the 5 wells of the ELISA plate, while distilled water is placed in one well as a blind test. In the other wells, the samples are placed and then 50 μL of 2N phenol foil is added to all of them and then they are read in two types of absorbances, 360 nm and 450 nm. The obtained values are recorded on the special paper of the apparatus (LOWRY 1990).

Results

The results of this research were calculated with the programs Minitab, Tuckey Kramer- ANOVA.

Average data of heavy metals Pb [mg/kg], Ni [μg/kg] and Zn [mg/kg], reported as dry weight values, in all sample types: soil and snail shells from Drenas and Opoja are reported in (Table 1).

Table 1

Summary table of average concentration of metals in soil and shell of samples analyzed in polluted locality Drenas

Metal/Soil	Concentration 1 km *A*	Concentration 2 km *B*	Concentration 5 km *C*	Significance
Pb	126.45 ±1.5 n.s	105.89 ±1.3*	89.46 ±0.9*	*A* : *B* n.s *B* : *C* < 0.05 *A* : *C* < 0.05
Ni	1053 ±3.2***	217.9 ±2.1*	522 ±0.42**	*A* : *B* < 0.001 *B* : *C* < 0.05 *A* : *C* < 0.01
Zn	188.3 ±1.8 n.s	132.3 ±1.6 n.s	145.8 ±1.7 n.s	*A* : *B* n.s *B* : *C* n.s *A* : *C* n.s
Metal/Shell	concentration 1 km *A*	concentration 2 km *B*	concentration 5 km *C*	significance
Pb	9.87 ±1.6**	31.16 ±2.2 n.s	50.75 ±2.5 n.s	*A* : *B* < 0.01 *B* : *C* n.s *A* : *C* < 0.01
Ni	0.42 ±0.9 n.s	0.17 ±0.2 n.s	0.29 ±0.8 n.s	*A* : *B* n.s *B* : *C* n.s *A* : *C* n.s
Zn	42.83 ±2.1 n.s	47.22 ±1.4 n.s	48.1 ±3.2 n.s	*A* : *B* n.s *B* : *C* n.s *A* : *C* n.s

From Table 1 we can see that for all three metals, when the concentration at 1 km was compared with 5 km, a significant $p < 0.05$ was recorded. While comparing 1 km with 2 km, then 2 km with 5 km, no significance was recorded.

Table 2

Summary table of metals in uncontaminated site Opoja

Soil/Shell samples control site				
Sample/metal	soil	SD	shell	SD
Pb [mg/kg]	9.23	0.9	0.09	0.02
Ni [mg/kg]	25.1	1.3	0.011 [µg/kg]	0.05
Zn [mg/kg]	46.2	0.62	0.025	0.09
Significant with pollution site	Pb, Ni, Zn 1–2–5 km; control $p < 0.001$		Pb, Ni, Zn 1–2–5 km; control $p < 0.001$	

The results from Table 2 show that in the analyzed soil samples, the concentrations of the three metals Pb, Zn, and Ni increase as they move away from the point of contamination. Also, the concentration of these metals has significant differences with the control samples. In our cases, we calculated the concentration of metal Ni by converting from mg/kg to µg/kg which recorded low values in shell samples.

According to Table 3 we can see that the value of Pb is below the standard limit, while the level of Ni exceeds 5 times the standard values in the soil. While Zn according to Table 3 standard is in the limit.

Table 3

Standard values according to European Directive 86/278/EEC

Metals	Soil [mg/kg of dry soil]		
	A	*B*	*C*
Arsenic (As)	30	55	80
Barium (Ba)	200	625	2000
Cadmium (Cd)	3	12	25
Chromium (Cr)	300	600	800
Cobalt (Co)	20	240	300
Copper (Cu)	200	300	500
Nickel (Ni)	300	600	800
Lead (Pb)	200	300	600
Mercury (Hg)	1.5	5	10
Molybdenum (Mo)	10	40	200
Tin (Sn)	20	50	300
Zinc (Zn)	300	500	1000
Selenium (Se)	2	100	200

From Table 3 of the locality control we see that the concentrations of metals have significant differences ($p < 0.001$) in all types of analyzed samples with contaminated site Drenas in our cases.

These heavy metals concentrations have also influenced the parameters of oxidative stress in the snail hepatopancreas, in which high values of protein carbonylation, malondialdehyde and total proteins were recorded, which are presented in Table 5 and Table 6 and graphical form.

Table 4

Table of average concentration of carbonyl proteins in polluted site Drenas

Hepatopancreas samples in three distances 1, 2, 5 km				
Samples	coordinates	weight [mg]	abs	conc. [µmol/L]
A1	North sample	0.397	0.170	70.4
B1	South sample	0.531	0.342	14.8
C1	North sample	0.621	1.400	62.9
D1	South sample	0.474	1.623	73.2
E1	North sample	0.527	0.675	30.3
F1	South sample	0.426	1.056	47.3
A4	North sample	0.472	1.592	716
B4	South sample	0.437	0.434	19.2
C4	North sample	0.533	0.345	15.4
D4	South sample	0.491	0.383	16.7
E4	North sample	0.413	0.318	13.8
F4	South sample	0.468	0.342	14.9
Average				91.24
STD				19.10

Table 5

Table of average concentration of carbonyl proteins in unpolluted site Opoja

Unpolluted site			
Sample	weight [mg]	abs	conc. [mol/L]
1	0.236	0.086	3.23
2	0.324	0.017	0.9
3	0.421	0.028	0.59
4	0.382	0.104	0.41
5	0.354	0.072	2.58
Average			1.54
SD			1.27

These results are presented graphically and we see that the highest concentration of protein carbonylation is higher in the southern part of the country than the northern part, these values correlate with the concentrations of metals which are also high in the southern part of the polluted area.

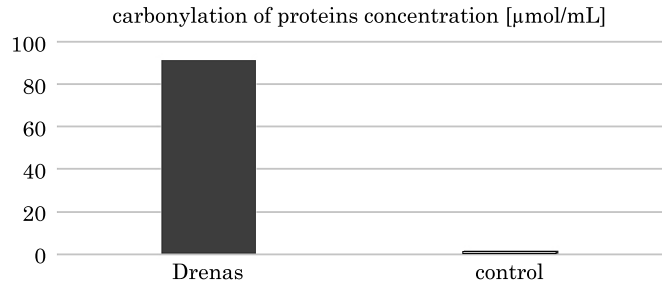

Fig. 2. Concentration of protein carbonylation in polluted area Drenas and unpolluted area control – Opoja

From Figure 1. we see that we have a highly significant difference $p < 0.001$ between the polluted and control area.

The concentration of these heavy metals has also influenced the high values of lipid peroxidation (MDA) and total proteins in snail hepatopancreas, these results are presented in Table 6 and Table 8.

Table 6

Summary table of average MDA in polluted site Drenas

Hepatopancreas samples in three distances 1, 2, 5 km of MDA				
No. of samples	coordinates	weight [mg]	abs	conc. [µmol/L]
A1	North sample	0.397	0.044	128
B1	South sample	0.531	0.119	176
C1	North sample	0.621	0.082	152
D1	South sample	0.474	0.073	46
E1	North sample	0.527	0.047	103
F1	South sample	0.426	0.104	166
A4	North sample	0.472	0.186	107
B4	South sample	0.437	0.239	153
C4	North sample	0.533	0.066	42
D4	South sample	0.491	0.206	132
E4	North sample	0.413	0.097	169
F4	South sample	0.468	0.230	147
Average				126.75
STD				44.9

Table 7

Summary table of average concentration of MDA in unpolluted site Opoja

Unpolluted site			
Sample	weight [mg]	abs	conc. [µmol/L]
1	0.236	0.065	7.1
2	0.324	0.053	4.2
3	0.421	0.038	1.9
4	0.382	0.103	6.5
5	0.354	0.092	2.5
Average			4.44
SD			2.07

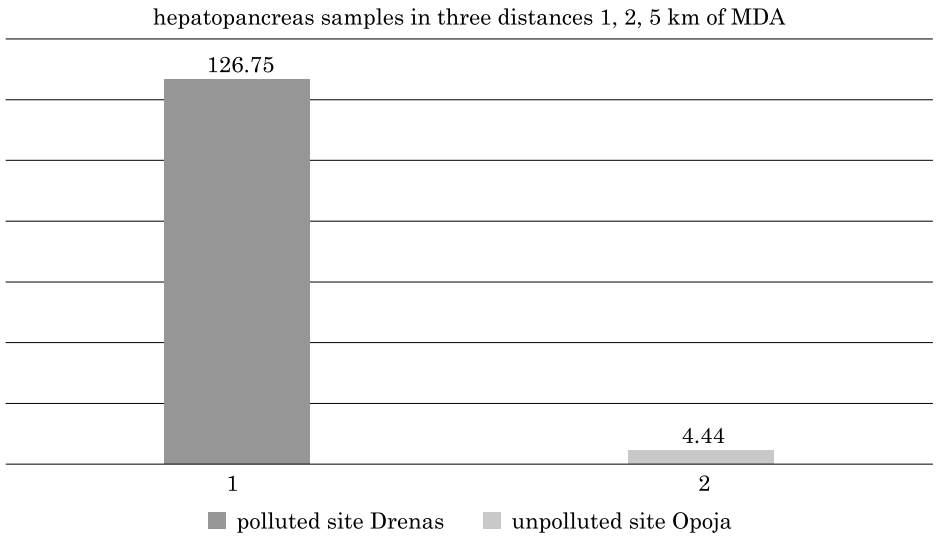

Fig. 3. Concentration of MDA µmol/L in polluted area Drenas and unpolluted area Opoja

Table 8

Summary concentration of total proteins in polluted site Drenas

Total proteins in hepatopancreas at polluted site Drenas					
Sample	weight [mg]	Total [ug/100 uL]	absorbance	450 nm	630 nm
1	2	3	4	5	6
A1O	0.397	23.25167	0.522	0.0696	0.0497
A4O	0.531	19.73274	0.443	0.0591	0.0422
B1O	0.621	18.44098	0.414	0.0552	0.0394
B4O	0.474	16.6147	0.373	0.0497	0.0355

cont. table 8

1	2	3	4	5	6
C1O	0.527	9.131403	0.205	0.0273	0.0195
C4O	0.426	12.82851	0.288	0.0384	0.0274
D1O	0.472	16.39198	0.368	0.0491	0.0350
D4O	0.437	20.62361	0.463	0.0617	0.0441
E1O	0.533	11.80401	0.265	0.0353	0.0252
E4O	0.491	22.49443	0.505	0.0673	0.0481
F1O	0.413	12.78396	0.287	0.0383	0.0273
F4O	0.468	29.66592	0.666	0.0888	0.0634
Mesatarja	–	17.81	–	–	–
STD	–	5.54	–	–	–

Table 9

Summary concentration of total proteins in unpolluted site Opoja

Control samples Brezne Opoja				
Sample	Total	abs	450 nm	630 nm
1	5.43	0.122	0.0163	0.0116
2	4.54	0.102	0.0136	0.0097
3	11.67	0.262	0.0349	0.025
4	8.69	0.195	0.026	0.0186
5	7.31	0.164	0.0219	0.0156
Average	7.53	significance		
STD	2.52			
D : B	Drenas: Brezne		$p < 0.05$	

Discussion

Our results show high concentrations of metals lead (Pb), zinc (Zn), nickel (Ni) at a distance of 5 km from the concentric circle in Drenas locality from the center of pollution "Ferronikel" mine. Also our research shows the influence of climatic and seasonal factors on the distribution of metals in the soil, since the measurements were made during the early summer-late autumn season. Measurements show high concentrations moving away from the source of pollution as well as in the southwest exposure of the country. These measurements correspond with those of other authors, according to VELIU et al. (2008), states that the air quality research in the vicinity of TC Kosova A has shown that the annual measure of lead (Pb) and zinc (Zn) concentrations compared to European standards are significantly higher.

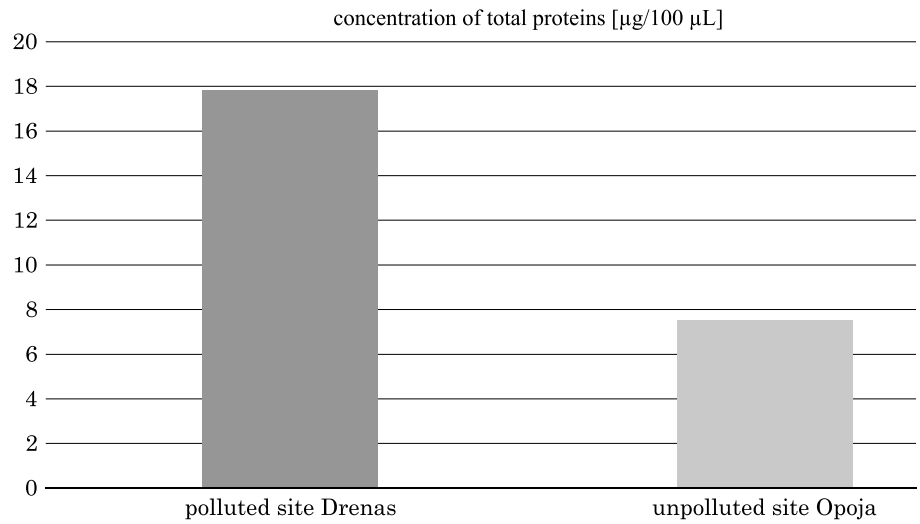

Fig. 4. Concentration of total protein in polluted and unpolluted site

Also, research by BAJRAKTARI et al. (2020) shows that pollution through coal fly ash in the industrial area of Thermal Power Plants (Kosova A and B) causes stratification of metals at different distances and their significant bioaccumulation from soil to snails. So this research also stated for pollution same as we did.

Study from RADWAN et al. (2010) shown that the maximum metal content in snail tissue is reached in autumn and winter and the lowest levels in summer.

The Roman snail (*H. pomatia*) is an important bioindicator of the accumulation of long-term exposure to metals in contaminated environments because of its remarkable capacity to concentrate heavy metal HMs in its body. The snail's hepatopancreas was discovered to be particularly sensitive to HM and frequently accumulated quantities of lead (Pb), nickel (Ni), zinc (Zn), copper (Cu), and cadmium (Cd) that were many times higher in the soil-snail chain link than at lower soil trophic levels. This organ can therefore be utilized to monitor the bioavailability of heavy metal HM in soil (NICA et al. 2012).

Research on this problem is few however, regarding the implication of these analyzed metals in oxidative stress, the results of our research are similar to those of other authors where according to ATAILIA et al. (2016) chronic exposure (5 mg/kg) of snails to the combination of Pb, Zn, Ni heavy metal dust cause changes in enzymatic activities and are also responsible for the development of oxidative stress, which is manifested by increased catalase activity and lipid peroxidation (MDA).

High activity of antioxidant enzymes (glutathione peroxidase (GPX), catalase, glutathione-S-transferase, and high levels of protein carbonylation and lipid peroxidation (MDA) have been recorded in the hepatopancreas of the snail *H. pomatia* (GUESSASMA et al. 2020).

Research by XIE et al. (2016) showed that there were differences in the modes of action of different metal ions in Lipid Peroxidation LPO. The reason for this phenomenon was that Reactive Oxygen Species ROS generated by heavy metal stress could not be removed over time, and the double bonds of unsaturated fatty acids in membrane phospholipids were attacked by excess ROS. Consequently, this resulted in lipid peroxidation, and the MDA content increased accordingly due to the heavy metals Pb and Cd.

Due to the creation of covalent bonds, which exist primarily between heavy metals and sulhydryl groups of the proteins, antioxidant enzymes can be inactivated as a result of direct binding of heavy metals to the active sites of the enzymes (ERCAL et al. 2001). Based on a research CANESI et al. (1999), revealed that the copper-induced increase in Glutathione S-transferases GST activity may be a result of an increased use of GSH in conjugation processes involved in the metabolism of lipid hydroperoxides and carbonyl compounds created by the peroxidation of cellular membranes caused by the copper.

Additionally, proteins play a key role in the structure of the cell. Also, they provide energy while under a lot of stress (RADWAN et al. 2008). In handled snails, the toxic effects of chemical substances such as heavy metals and pesticides may result via increased energy consumption and/or organelle disintegration, which may promote protein production (EL GOHARY et al. 2021).

The importance of knowing these mechanisms is demonstrated by research LIU et al. (2022) which states that the contribution of Cd and Pb in their interactions varies with the dose and duration of exposure in the hepatopancreas of Macrobrachium nipponense. Cadmium (Cd) and lead (Pb) contribute equally to their interaction effects, regardless of concentration. According to recent study demonstrated that zinc oxide nanoparticles (ZnO NPs) have toxic effects on *Helix aspersa*, posing great challenges to the environment. Therefore, the misuse of nanomaterials may have relevant and negative effects on the environment and human health. Industrial applications of ZnO NPs need to be monitored and regulated. Helix aspersa is an excellent bioindicator of nanoecotoxic efficacy (ABDEL-AZEEM et al. 2021).

According to the health risk assessment, infants and children under the age of ten have significant non-carcinogenic hazards associated with

lead consumption. There is an urgent need for appropriate control and protection techniques to stop pregnant women, kids, and newborns from being exposed to lead (PERERA et al. 2021). The issue of metals in the environment poses a significant threat to both human and ecological health, and their removal from the environment is a worldwide topic that requires attention (KUMAR et al. 2022).

Conclusions

Based on our results, we can conclude that the activity of energy production from the drilling of soil from activity of minning "Ferronikel" in the Municipality of Drenas causes pollution with heavy metals specially for Ni with high concentrations above the rate allowed according to EU standards.

H. pomatia snails bioaccumulate these metals from the soil and plants in shell that grow in these areas, therefore they can serve as efficient bioindicators in environmental pollution.

These metals accumulated in the shell cause oxidative stress in the hepatocytes of the hepatopancreas in snail and may represent a problem in the public health of people since it is used for food and for trophic level cycle.

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