Carotenoids and Antioxidant Enzymes as Biomarkers of the Impact of Heavy Metals in food Chain

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ABSTRACT

Antioxidant enzymes (catalase and peroxidase) and carotenoids (lutein and â-carotene) are often used as biomarkers of metal contamination of water and agricultural soils. In this study, the effects of heavy metals present in irrigation water on the aforementioned carotenoids of potatoes (*Solanum tuberosum* L.) and carrots (*Daucus carota* L.), cultivated in a greenhouse and irrigated with a water solution including different levels of Cr(VI) and Ni(II) were investigated. These results were compared to the levels of the same metabolites that had been assessed in market-available potato and carrot samples. The findings indicated that the levels of the examined metabolites on the treated with Cr and Ni samples, resemble the levels of the same parameters in the market samples, originating from polluted areas. Therefore, the antioxidant enzymes, catalase and peroxidase, and the carotenoids, lutein and â-carotene, could be handled as indicators of heavy metal pollution.

Key words: Heavy Metals, Food Tubers, Antioxidant
Enzymes, Carotenoids, Biomarkers.

INTRODUCTION

Soil and water quality have a direct impact on the quality of our environment and consequently on our nutrition and health.[1] Environmental pollution due to anthropogenic operations such as sewage sludge, mining, industrial and domestic wastewater or excessive application of fertilizers and pesticides may lead to bioaccumulation of heavy metals in crops[2] and thus to serious cross-contamination of the food chain.[3] Heavy metal contamination of crops, such as potatoes and carrots, which are principal components of our daily diet, is a matter of great concern, as their consumption may result in accumulation of risk elements in the human body which can cause serious health problems[4]. However, the US and EU legislation for heavy metals in food is inadequate (i.e. only four heavy metals are regulated under current EU legislation, EC 1881/2006); whereas the respective legal limits for water are harsh.[5]

The use of biomarkers as indicators of the pollutant effect on an ecosystem has been widely studied in order to gain knowledge on the physiological or biochemical response of an organism to pollutant exposure.[6,7] Measurements of biochemical responses to pollutants may serve as early signals of biologically significant toxic exposure given that toxic effects tend to appear at the

subcellular level before appearing at higher levels of biological organisms.[8]

Biomarkers, such as enzyme activity and carotenoid levels could be used as indicators of the oxidative stress caused by heavy metal pollution. Therefore their assessment is more relevant biologically, when compared to trace element analysis. Furthermore, the analysis of these secondary metabolites has three distinct advantages, being swift and of low cost and not requiring expensive instrumentation as opposed to the techniques that are commonly used for the determination of heavy metal concentrations.

Previous studies of our team investigated the cross-contamination of the food chain caused by the environmental pollution in Asopos land (one of the biggest industrial areas of central Greece) by heavy metals; the levels of Ni and Cr were significantly higher in crops from Asopos area than in plants grown in areas besides water-bed pollution (control samples).[3,5] Another study of our team on the effects of these trace elements on the carotenoids and the antioxidant activity (DPPH) of carrots, potatoes and onions (*Allium cepa* L.) showed that the levels of â-carotene in carrots and the levels of lutein in potatoes from the Asopos area were significantly lower as opposed to the control samples.[9] These

two researches were the incentive of a greenhouse experiment, conducted by our team where the open-field irrigation conditions of Asopos area and Messapia region (Evia, Greece) were simulated. Both these regions have considerable levels of Cr and Ni in the underground water. The uptake of these heavy metals by carrots, potatoes and onions was studied and the resulting cross-contamination by the irrigation water of the potatoes and onions was screened, but no cross-contamination was observed for carrots[10].

In the present study, our scope was three-fold: 1) to examine the effects of Cr(VI) and Ni(II) in irrigation water on the carotenoid content (lutein and â-carotene) and the activities of antioxidant enzymes (catalase and peroxidase) in potatoes and carrots, cultivated in a greenhouse whilst being irrigated with Cr(VI) and Ni(II) contaminated water, 2) to compare the results of 10 to findings in the respective crops, bought from the local market and developed in areas with or without water-bed pollution and 3) to investigate the possibility of using the aforementioned parameters as biomarkers of heavy metal pollution.

MATERIALS AND METHODS

Experimental Design
Plants' Cultivation in a greenhouse

For the purposes of this study, potatoes and carrots were planted in a greenhouse (Harokopio University, Athens, Greece), at which the installation had four lines with irrigated water (i.e. four 300 L plastic tanks, four pumps, and four series of two tubs per series) was used. The cultivation duration was four months (September 2012 to January 2013). The four irrigation lines contained various levels of Cr(VI) and Ni(II), as follows: 0 ìg L^{-1} (control), 100 ìg L^{-1}, 250 μg L^{-1} and 1,000 μg L^{-1}. The solutions were prepared by solid $K_2Cr_2O_7$ and $NiCl_2.6H_2O$, diluted in tap water. All the plants were fertilized once every month (15N-30P-15K fertilizer, 2-4 g dry solids/plant diluted in water, depending on plant size) and irrigated every 3-10 days (depending on soil humidity). They were also supervised by a professional agriculturalist. The amounts of the main characteristics of control (clean) water were: pH=8.3, turbidity=7.6 NTU, EC=295 ìS cm^{-1}, TDS=156 mg l^{-1},

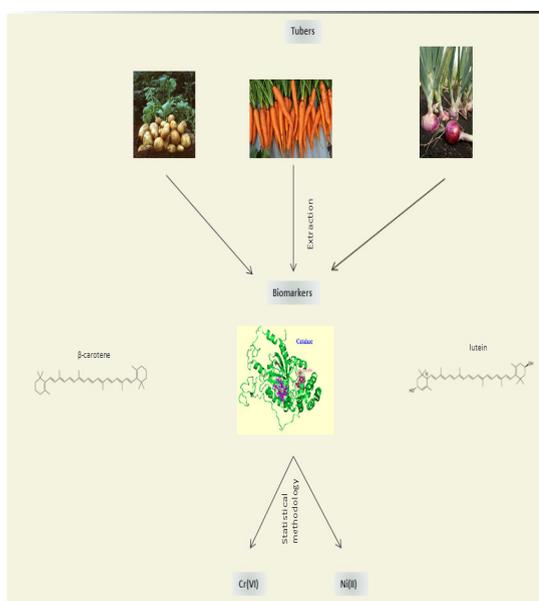**Fig.1: Picture of the Experimental Design**

Na=4.6 mg l^{-1}, K=0.8 mg l^{-1}, Pb=0.3 mg l^{-1}, Hg=0 mg l^{-1}, Cd=0 mg l^{-1}, Cr=0 mg l^{-1}.

Market Samples

Commercially available samples (potatoes and carrots, five different samples of each vegetable) originating from Greece and from other European countries (Holland and Cyprus) were obtained from supermarkets in Athens, Greece, the same period, when the greenhouse's vegetables were analyzed.

Enzyme Assay

The materials used for catalase and peroxidase assay were potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), ethylenediaminetetraacetic acid (EDTA), guaiacol, hydrogen peroxide (H_2O_2), plastic and quartz cuvettes (all from Sigma-Aldrich, Germany) and double distilled water (DDW).

Enzyme Extraction

Potatoes and carrots were cut into small pieces and homogenized with phosphate buffer (50 mM, pH 7.0) containing 1 mM of EDTA. The homogenate got filtered through and the obtained extract was centrifuged at $7000 \times g$ for 20 minutes at 4 °C. The clear supernatant was kept at 0-4 °C in 5 mL vials and was suitably thawed before the enzyme analysis[11].

Table 1: Levels of catalase, peroxidase, lutein and β-carotene (Levels of catalase and peroxidase in Δg^{-1} min^{-1} fresh weight, lutein and β-carotene in μg g^{-1} fresh weight. Potato and carrot samples irrigated with water solution containing Cr(VI) and Ni(II) in concentrations ranging from 0 to 1000 μg L^{-1})

Parameter	0 μg L^{-1}	100 μg L^{-1}	250 μg L^{-1}	1000 μg L^{-1}
Catalase (p)[a]	0.160 ± 0.047	0.167 ± 0.029	0.138 ± 0.097	0.162 ± 0.031
Peroxidase (p)[a]	2.075 ± 0.171	7.125 ± 1.769	3.625 ± 2.42	4.350 ± 0.645
Lutein (p)[a]	0.080 ± 0.001	0.312 ± 0.049	0.024 ± 0.014	nd
Catalase (c)[b]	0.088 ± 0.024	0.054 ± 0.014	0.046 ± 0.027	0.052 ± 0.004
Peroxidase (c)[b]	0.675 ± 0.126	0.275 ± 0,096	0.775 ± 0.299	0.325 ± 0.126
Lutein (c)[b]	6.117 ± 2.620	4.608 ± 0,525	6.172 ± 1.450	2.015 ± 0.325
β-Carotene (c)[b]	38.603 ± 12.080	57.588 ± 14,710	58.895 ± 34.320	31.598 ± 7.290

Results are expressed as mean ± standard deviation (n=3).
a: potato samples; b: carrot samples; nd: not detected

Table 2: Two by two" comparisons of the enzyme activity and carotenoid content of potatoes and carrots

μg L^{-1} Cr & Ni		p-value						
		Catalase		Peroxidase		Lutein		β-Carotene
		Potatoes	Carrots	Potatoes	Carrots	Potatoes	Carrots	Carrots
0	100	0.886	0.029[a]	0.029[a]	0.029[a]	0.029[a]	0.229	0.629
	250	0.686	0.057[b]	0.343	0.686	0.029[a]	0.629	0.400
	1000	0.606	0.029[a]	0.029[a]	0.029[a]	-	0.057[b]	0.057[b]
100	250	0.343	1.000	0.057[b]	0.029[a]	0.029[a]	0.114	0.686
	1000	0.686	1.000	0.057[b]	0.686	-	0.029[a]	0.029[a]
250	1000	0.486	0.629	0.886	0.057	-	0.029[a]	0.029[a]

a: statistically significant (p<0.05) (Mann-Whitney non parametric test)
b: marginally significant (0.05<p<0.1) (Mann-Whitney non parametric test)

Enzyme Analysis

Catalase (CAT E.C.1.11.1.6) activity ($\ddot{A}\acute{A}_{240}$ $min^{-1} g^{-1}$ FW) assay was based on the absorbance at 240 nm on the UV spectrophotometer. The decrease in absorbance was recorded over a period as described previously.[12] A complete reaction mixture was including 1.5 mL of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mL of 75 mM H_2O_2, 0.2 mL of enzyme extract and 0.8 mL of DDW, in quartz cuvettes.

Peroxidase (POX E.C.1.11.1.7) activity ($\ddot{A}\acute{A}_{470} min^{-1} g^{-1}$ FW) was assayed as an increase in optical density because of the oxidation of guaiacol to tetraguaiacol[13]. The complete reaction mixture contained 1.0 ml of 100 mM potassium phosphate buffer (pH 6.1), 0.5 ml of 96 mM guaiacol, 0.5 mL of 12 mM H_2O_2, 0.1 ml of enzyme extract and 0.4 ml of double distilled water (DDW). The enzyme activity was measured at 470 nm, in plastic cuvettes, for 1 min on a UV spectrophotometer.

Every plant sample was analysed in triplicate High Performance Liquid Chromatography (HPLC) Assay

All materials were obtained from Merck, Germany except of lutein which was obtained from Extrasynthese, France and â-carotene which were obtained from Fluka, UK.

Sample Preparation

All plant samples were washed thoroughly with double distilled water (DDW) and were peeled and carved afterwards. Then, they were ground to obtain a thin powder using a food processor with stainless steel cutters and then stored at −15 °C before the assay. The extraction of carotenoids was performed as described before[14] with minor modifications. More specifically, carotenoids were released from the powdered samples (5g) by adding 0.5 g of magnesium carbonate and 25 mL of 50:50 methanol:tetrahydrofuran (THF). The crude suspensions were centrifuged at 6000 × g at 4 °C for 10 min (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments). The process was repeated until the extracting solvent was colorless and the extract was vacuum filtered thereafter. The filtrates were then concentrated using a rotary evaporator apparatus (Heidolph Laborota 4000 eco/WB/G1) and transferred to a 25 mL volumetric flask, while carrot extracts were diluted to a known volume with methanol so that THF levels were less than 10% of the entire solution. Before the injection into the HPLC analysis system, all samples were filtered through a 0.45 ìm filter. All analyses were done in triplicate.

Preparation of Calibration Curves

Five mg of lutein were dissolved in 100 mL reagent alcohol containing 30 mg L^{-1} butylated hydroxytoluene (BHT), while 5 mg of β-carotene were dissolved in 10 mL THF (1 l of THF previously stabilized with 250 mL BHT) and then diluted to 100 mL with reagent alcohol. Full standard curves were constructed with five different concentrations for each carotenoid. The concentrations for the lutein standards were from 0.01 to 0.16 mg L^{-1}, while the β-carotene concentrations were from 0.5 to 10.0 mg L^{-1}, as described before[15]. All analyses of standard solutions were performed in triplicate.

The calibration curves obtained (y being the peak area, x being the concentration of the standard compound in mg L^{-1}) were y = 1236560.31

Table 3: Catalase, peroxidase and lutein levels in potato market samples originating from Greece and Cyprus. (Levels of catalase and peroxidase in $\Delta g^{-1} min^{-1}$ fresh weight and lutein in µg g^{-1} fresh weight)

Parameter	Achaia (GR)	Evia (GR)	Cyprus	Orhomenos BIO(GR)	Naxos (GR)
Catalase	0.131 ± 0.016	0.167 ± 0.056	0.037 ± 0.006	0.127 ± 0.038	0.041 ± 0.014
Peroxidase	5.180 ± 0.075	8.600 ± 0.351	6.650 ± 0.050	4.150 ± 0.350	3.930 ± 0.225
Lutein	0.033 ± 0.029	nd	Nd	0.245 ± 0.102	0.041 ± 0.011

Results are expressed as mean ± standard deviations (n=3). nd: not detected

x + 3574.21 with R^2 = 0.9999 for lutein and y = 1559668.43 x − 165770.72 with R^2 = 0.9986 for â-carotene.

HPLC Analysis

The HPLC apparatus consisted of an Agilent SERIES 1100, equipped with a Variable Wavelength UV-Vis detector and an integrator Agilent 3395. A 50 µl loop was used. All solvents had purity of HPLC grade. The mobile phase was a binary mixture of methanol : acetonitrile (85:15, v/v). The column was a C18 Zorbax SB (4.6 × 250 mm, 5 ìm). The column was kept at room temperature (20 °C) and the flow rate was 1.5 ml min^{-1}. The detection wavelength was 450 nm.

Statistical methodology

All analyses were carried out in triplicate and the results were statistically edited using IBM SPSS 19.

The Mann-Whitney non-parametric test was applied for the "two by two" comparisons between the groups of plants irrigated with different concentrations of heavy metals (Cr, Ni) in relation to their metabolites (catalase, peroxidase, lutein and â-carotene).

Therefore, each group of plants irrigated with the same metals concentrations (plants grown in the same tub or plants grown in the same geographical area) was compared with each and every other group of plants irrigated with other concentrations (e.g. 0 and 100 ìg/l, 0 and 250 ìg/l, Naxos and 0 ìg/l, etc.) to find out if there were statistically significantly differences in the metabolites of the different groups.

The comparisons between the groups of plants were based on median values of metabolites. The choice of the median values, instead of mean values, was made because, in many occasions, the distributions of values diverged from the normal distribution.

For the total growing season (September 2012 – January 2013) each tuber was irrigated with approximately 40 L of water with Cr(VI) and Ni(II) concentrations varying from 0 to 1000 µg L^{-1}. The range of the metal concentrations was chosen in order to resemble the concentration levels of Cr(VI) and Ni(II) that have been detected in the underground waters of Asopos area and Messapia and thus, to simulate as close as possible these open-field cultivation conditions[3]. The total mass of Cr(VI) and Ni(II) added in each tub, for the irrigation watering line of 0, 100, 250, and 1,000 µg L^{-1}, was 0, 4,000, 10,000, and 40,000 ìg, respectively. No symptoms of toxicity were observed in any plant. The antioxidant enzyme and the carotenoid response of potato and carrot samples to heavy metal treatment are described in Table 1, while the results of statistical analysis are shown in Table 2.

CAT activity in potato samples did not show any statistically significant differences, whereas POX activity has increased statistically significantly in potato samples irrigated with water containing 100 ìg L^{-1} (+243.4 percent in median value) and 1000 ìg L^{-1} (+109.6 percent in median value) of Cr(VI) and Ni(II) as opposed to control samples. A marginally significant decrease was observed in potato crops irrigated with water containing 250 µg L^{-1} (-56.1 percent in median value) and 1000 µg L^{-1}

Table 4: Catalase, peroxidase, lutein and β-carotene levels in carrot market samples originating from Greece and Holland. (Levels of catalase and peroxidase in Δg-1 min^{-1} fresh weight and lutein and β-carotene in µg g-1 fresh weight).

Parameter	Evia (GR)	Avlonas (GR)	Holland BIO	Viotia (GR)	Marathon (GR)
Catalase	0.081 ± 0.012	0.037 ± 0.025	0.104 ± 0.073	0.008 ± 0.002	0.087 ± 0.048
Peroxidase	0.400 ± 0.125	0.400 ± 0.050	0.175 ± 0.025	0.025 ± 0.075	0.250 ± 0.100
Lutein	6.600 ± 1.000	5.610 ± 1.980	7.650 ± 1.912	5.150 ± 0.345	5.490 ± 0.139
β-Carotene	52.310 ± 0,568	64.660 ± 16.165	59.500 ± 14.875	52.500 ± 0.490	55.150 ± 11.878

Results are expressed as mean ± standard deviations (n=3).

(-38.9 percent in median value) of Cr(VI) and Ni(II) in respect to potato samples irrigated with 100 μg L^{-1}.

The increased activity of POX suggests strong induction of oxidative stress as antioxidant enzymes constitute part of the detoxification mechanisms in plants[16]. These high levels of the antioxidant enzyme activity may have been an immediate reply to the generation of superoxide radicals by Cr-induced blockage of the electron transport chain in the mitochondria[17].

CAT activity in carrot samples irrigated with water containing 100 μg L^{-1} and 1000 μg L^{-1} Cr(VI) and Ni(II) decreased statistically significantly (-38.6 percent and -47.7 percent in median value, respectively) as correlated to the control ones. POX activity in carrot crops irrigated with water containing 100 μg L^{-1} Cr(VI) and Ni(II) decreased statistically significantly as compared to the control samples and the ones irrigated with water containing 250 ìg L^{-1} Cr(VI) and Ni(II) (-59.2 percent in median value and -64.5 percent in median value, respectively). A statistically significant decrease was also noticed in the carrot crops irrigated with water containing 1000 μg L^{-1} in respect to the control ones (-51.8 percent in median value), whereas the decrease was marginally significant as compared to the carrot samples irrigated with water containing 250 μg L^{-1} Cr(VI) and Ni(II) (-58.1 percent in median value).

Low CAT and POX activities result in an increase of H$_2$O$_2$ levels, causing oxidative stress, inactivation of CAT and finally blockage of the synthesis of new enzyme[18]. CAT is naturally present in peroxisomes, where it catalyzes the disintegration of the H$_2$O$_2$ produced during photorespiration[19]. A previous study on carrots treated with excess Cr found decreased CAT activity in carrot leaves[20], possibly as a result of the absolute or partial substitution of Fe from active sites or due to low levels of Fe in leaves inhibiting the embodiment of Fe in porphyrin of the enzyme[21-23]. Furthermore, the decreased activity of POX may have occurred as a consequence to peroxidative damages of the thylakoid membrane[24].

Lutein content in potato crops irrigated with water containing 100 μg L^{-1} Cr(VI) and Ni(II) increased statistically significantly as opposed to the control ones (+290 percent in median value), while lutein levels in potato samples irrigated with water containing 250 μg L^{-1} Cr(VI) and Ni(II) decreased statistically significantly as compared to the control samples and the ones polluted with water containing 100 μg L^{-1} Cr(VI) and Ni(II) (-70 percent in median value and -92.3 percent in median value, respectively). Lutein levels in potato crops irrigated with water including 1000 ìg L^{-1} Cr(VI) and Ni(II) were not detectable.

â-Carotene and lutein levels in carrot crops irrigated with water containing 1000 ìg L^{-1} Cr(VI) and Ni(II) decreased marginally significantly as opposed to the control ones (-18.1 percent, in median value and -67 percent in median value, respectively), whereas the decrease was statistically significant in respect to the â-carotene and lutein content of the carrot samples irrigated with water containing 100 ìg L^{-1} Cr(VI) and Ni(II) (-45.1 percent in median value and -56.3 percent in median value, respectively) and 250 μg L^{-1} Cr(VI) and Ni(II) (-46.3 percent in median value and -67.4 percent in median value, respectively).

Chromium toxicity can cause metabolic disturbances such as transitions in pigment production, augmentation of metabolite production (ascorbic acid, glutathione) and the generation of new metabolites as a reinforcement of the detoxification mechanism of the plant[17], while nickel can provoke suppression in growth and reduction of the Fe levels[24]. Even if an amount of these trace elements can be filtered or rejected through the plant root tissues, higher levels of heavy metals in the soil are affiliated to virulent effects[25].

As has been observed earlier for potato and carrot crops grown in Asopos region (where the underground waters are contaminated by heavy metals), heavy metals may induce the biosynthesis of lutein and â-carotene. Lutein and â-carotene levels in Asopos potatoes and carrots, respectively, decreased as compared to the carotenoid levels in the control samples, suggesting an impact of heavy metals in carotenoid metabolic pathway[9].

The results of carotenoid and antioxidant enzymes analysis of potato and carrot market

samples are shown in tables 3 and 4, while the results of the statistical analysis of the samples are visible in the tables 5 and 6.

Given the statistically significant and the marginally significant differences which have emerged from the comparison between the same biomarkers in the two researches, the following conclusions could be derived.

The potato samples originating from Achaia (Greece) simulate with the potato samples irrigated with water containing 100 – 1000 μg L^{-1} Cr(VI) and Ni (II), while the potato samples originating from Orhomenos (Viotia, Greece, organic farming) and the ones from Naxos (Greece) simulate with the potato samples which were irrigated with

water containing 250– 1,000 ìg L^{-1} Cr (VI) and Ni (II) and 250 μg L^{-1} Cr(VI) and Ni(II), respectively. Furthermore, the potato samples originating from Evia (Greece) appeared to be similar with the potato samples irrigated with water including 100 μg L^{-1} Cr (VI) and Ni (II). However, the potato samples originating from Cyprus do not simulate with any of the potato samples cultivated in the greenhouse.

The carrot samples originating from Holland (organic farming) simulate with the carrot samples of irrigated water's concentration about 100 μg L^{-1} of Cr (VI) and Ni (II), whereas the carrot samples originating from Avlona (Greece) simulate with the carrot samples irrigated with water containing 100– 1,000 μg L^{-1} Cr (VI) and Ni (II). The carrot samples

Table 5: Two by two" comparisons of the enzyme activity and the carotenoid content in potatoes

Potato Market samples from Greek areas and Cyprus	μg L^{-1} Cr & Ni	Catalase (p-value)	Peroxidase (p-value)	Lutein (p-value)
Achaia	0	0.486	0.029[a]	0.057[b]
(GR)	100	0.343	0.343	0.057[b]
	250	1.000	0.343	1.000
	1000	0.486	0.200	-
Evia	0	0.343	0.029[a]	-
(GR)	100	0.886	0.343	-
	250	0.686	0.029[a]	-
	1000	0.686	0.029[a]	-
Cyprus	0	0.029[a]	0.029[a]	-
	100	0.029[a]	0.686	-
	250	0.029[a]	0.057[b]	-
	1000	0.029[a]	0.029[a]	-
Orhomenos	0	0.686	0.029[a]	0.057[b]
BIO (GR)	100	0.343	0.029[a]	0.400
	250	0.886	1.000	0.057[b]
	1000	0.486	1.000	-
Naxos	0	0.029[a]	0.029[a]	0.057[b]
(GR)	100	0.029[a]	0.029[a]	1.000
	250	0.057[b]	1.000	0.057[b]
	1000	0.029[a]	0.200	-

a: statistically significant difference (p<0.05) (Mann-Whitney non parametric test) b: marginally significant difference (0.05<p<0.1) (Mann-Whitney non parametric test)
-: no samples for comparison

originating from Evia (Greece) and the ones from Marathon (Greece) simulate with the carrot samples irrigated with water containing 0 – 250 µg L^{-1} Cr (VI) and Ni (II) and 100 ìg L^{-1} Cr (VI) and Ni (II), respectively. Finally, the carrot samples originating from Viotia (Greece) do not simulate with any of the carrot samples cultivated in the greenhouse.

DISCUSSION

The results of this study on the carotenoid (lutein and â-carotene) substance and the activity of antioxidant enzymes (catalase and peroxidase) on Cr and Ni treated potatoes and carrots show a clear correlation of Cr and Ni in irrigation water and these secondary metabolites. Moreover, the levels of

the same parameters on potato and carrot samples cultivated in areas with water bed pollution simulate the ones on potato and carrot samples irrigated by Cr and Ni solutions. Hence, these secondary metabolites might serve as indicators of heavy metal pollution in water beds and plants.

Given that potatoes and carrots are considered staple foods and they are often used in baby food, contamination by heavy metals of these food tubers not only reduces their nutritional value but also causes a considerable risk. Therefore, legal limits of heavy metals in food need to be revised and introduced by the competent legislative authorities, worldwide.

**Table 6: Two by two" comparisons of the enzyme acti
vity and the carotenoid content in carrots**

Carrot Market samples from Holland and Greek areas	µg L^{-1} Cr & Ni	Catalase (p-value)	Peroxidase (p-value)	Lutein (p-value)	β-Carotene (p-value)
Evia (GR)	0	0.868	0.057[b]	0.343	0.114
	100	0.114	0.200	0.057[b]	0.343
	250	0.114	0.114	1.000	0.343
	1000	0.057[b]	0.486	0.029[a]	0.029[a]
Marathon (GR)	0	0.686	0.029[a]	0.343	0.114
	100	0.686	0.886	0.114	1.000
	250	0.343	0.029[a]	0.486	0.686
	1000	0.200	0.486	0.029[a]	0.029[a]
	0	0.886	0.029[a]	0.724	0.886
Holland BIO	100	0.343	0.200	0.686	0.057[b]
	250	0.029[a]	0.029[a]	0.400	0.486
	1000	0.200	0.114	0.343	0.029[a]
Avlonas (GR)	0	0.057[b]	0.029[a]	0.686	0.200
	100	0.486	0.114	0.343	0.343
	250	1.000	0.114	1.000	0.686
	1000	0.343	0.343	0.343	0.343
Viotia (GR)	0	0.029[a]	0.029[a]	0.400	0.114
	100	0.029[a]	0.486	0.629	0.343
	250	0.200	0.029[a]	0.229	0.343
	1000	0.029[a]	0.343	0.057[b]	0.029[a]

a: statistically significant difference (p<0.05) (Mann-Whitney non parametric test)

b: marginally significant difference (0.05<p<0.1) (Mann-Whitney non parametric test)

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