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# HEAVY METAL CONTENT IN ENVIRONMENTAL ENRICHMENT USED FOR LABORATORY RODENTS

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**Summary.** Laboratory animals should be kept in facilities ensuring appropriate environmental conditions for a given species, including the necessary freedom of movement, food and water and care to ensure their health. No lesser of an important factor in improving animal welfare is through the use of various types of elements enriching the environment, which do not disturb proper behavioural attitudes and reduce stress factors. Such enrichment includes commercially available elements made of polycarbonate, cotton, aspen wood and cellulose and paper tubes from households of an economic origin. Commercially available enrichments have a high standard of manufacturing and guarantee safety, which, in the case of "home-made" cellulose tubing, is difficult to guarantee, particularly when they contain dyes, printing inks or adhesive residues. The aim of the study was to assess the content of heavy metals in home-made environmental enrichments. The comparative material was commercially available enrichments. Two methods of mineralisation of the tested material and ICP-OES analytical method were used to determine the content of heavy metals. Nutritional intake standards for rodents served us as critical parameters. The results of the analysis showed that both types of environmental enrichment ("home-made" and "commercial") do not contain toxic heavy metals. The only impurities that were determined by the method used were iron and copper, whose content in the analysed material differed depending on the method of sample preparation. The content of copper and iron was much lower than the feeding standards for rodents.

**Key words:** heavy metals, enrichment, laboratory animals, paper tubes, contamination

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## INTRODUCTION

Heavy metals are naturally occurring elements that have a relatively high atomic mass, atomic number and density. They are non-removable from the environment, which means that once introduced into the environment they circulate constantly, changing at most their chemical form. Heavy metals participating in life processes include: macroelements such as calcium (Ca), magnesium (Mg) and potassium (K) and a wide range of microelements such as iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), cobalt (Co), molybdenum (Mo), selenium (Se), chromium (Cr), silicon (Si), vanadium (V), tin (Sn), nickel (Ni), aresene (As) [Dobrzański 1996]. It is worth noting that heavy metals also include elements that do not take part in metabolic transformations and can have embryotoxic, teratogenic, mutagenic and carcinogenic properties. This group includes: mercury (Hg), lead (Pb), thallium (Tl), barium (Ba) or cadmium (Cd) [Kupczyk 1995, Ociepa-Kubicka 2012, Sowa-Lewandowska 2013]. In the interest for the proper functioning of the animal organism, standards were established for the presence of undesirable substances in feeding stuffs. These values for heavy metals are expressed in $mg \cdot kg^{-1}$ (ppm) for feed with specific moisture content. However, it should be remembered that heavy metals can be introduced into the animal body not only with feed but also with water, litter or enrichment [Kinal 2009].

In the interest of laboratory animal welfare [Broom 1987] and in accordance with the guidelines laid down in Directive 2010/63/EU on the protection of animals used for scientific purposes, environmental enrichment shall be introduced into cages in order to provide the animals with the highest possible level of behavioural comfort. A very wide range of commercial environmental enrichment elements is available, including polycarbonate houses and tubes, cotton swabs, wooden blocks and cardboard tubes. In parallel to this enrichment, many animal facilities use paper tubes from households, such as toilet paper or paper towels. While the commercially available environmental enrichments are characterised by a high standard of workmanship and a guarantee of safety, there is still a discussion about the quality and safety of the “home-made” cellulose tubing. Household tubes are very likely to contain dyes, printing inks or adhesive residues that may carry heavy metals that mainly come from the manufacturing process. This means that animals, by playing, biting or shredding such enrichment into nesting material, may introduce into the body some amounts of harmful heavy metals, which may cause their health to deteriorate and adversely affect the quality of research results.

Therefore, enrichments used in animal houses (commercial and “home-made”) for heavy metal content were tested. The following undesirable heavy metals were selected for determination: Cd, Pb, Co, As and Hg as well as two elements belonging to microelements participating in life processes: Cu and Fe.

## MATERIAL AND METHODS

### Materials

The research material consisted of seven different enrichments. The first group of materials included professional enrichments for rodents such as: wooden blocs made of aspen wood (*Populus tremula*), resin-free (W1), cotton cocoons (W2) and cardboard tunnel (W3). The second group of materials consisted of “home-made” enrichments from toilet paper rolls, containing and not containing printing ink (W4 and W5) and rigid and soft rolls from paper towels (W6 and W7). The “home-made” research material was selected randomly from our facility resources and was autoclaved to represent routinely placed enrichments in cages.

### Preparation of samples for analysis

Six samples were prepared from each research material and divided into two groups ($2 \times n = 3$). The cardboard tubes were cut into 1 × 1 cm pieces, which weighed 0.44–0.53 g each. The cotton cocoons and wooden blocks were used to prepare samples of a similar weight to the cardboard samples (about 0.5 g). The first group of samples ($n = 3$) was subjected to a migration test (Method A) used in the analysis of food packaging. The samples were placed in glass penicillin tubes with a 3% acetic acid solution and kept in an incubator at 40°C for 48 h [Tye and Engel 1965]. The second group of samples was subjected to wet oxidative mineralisation (Method B) [Conti 1997]. The samples were placed in glass vials in a perhydrol solution with 60% perchloric acid (1 : 1, v/v) and heated in a water bath at 75°C for 2 h. Additionally, a matrix was prepared, i.e. samples without the tested material.

All the samples before entering the spectrometer were filtered to obtain a clear solution and then diluted hundredfold with 0.5M nitric acid.

### Analysis of heavy metals content

The analysis of samples for heavy metals content was performed with the ICP-OES method (optical coupled plasma – optical emission spectrometer ICP-OES) on a Perkin Elmer Optima 7300 DV spectrometer. For each examined element the limit of quantification (LOQ) was determined and the quantitative content of heavy metals in the samples was calculated using calibration curves. Nutrient standards for rodents developed by the National Research Council (US) Subcommittee on Laboratory Animal Nutrition, determining, among others, the daily demand for microelements, served us as critical parameters [National Research Council 1995].

### Statistical methods

Statistical analysis includes the calculation of averages and standard deviation for the results of determined elements content in the prepared samples. The results from two methods (wet mineralisation, migration test) were compared using the Student’s t-test. The calculations were performed using the GraphPad Prism 8 software.

## RESULTS AND DISCUSSION

The daily consumption of cardboard tubes was analysed to determine the potential daily exposure of mice and rats to the heavy metals. The results are presented in Table 1.

Using reference curves based on standards, limit of quantification (LOQ) was determined for individual heavy metals. The results are presented in Table 2.

Tables 3 and 4 show the results of the heavy metals content analysis in the examined environmental enrichments expressed as µg of metal in 1 g of material. The tables present the results for the samples prepared with the migration test method (A) and the mineralisation method (B) respectively.

Table 1. Daily consumption of cardboard components by mice and rats ($n$ = 6, mean ±*SD*)

Tabela 1. Codzienne zużycie elementów tekturowych przez myszy i szczury ($n$ = 6, średnia ±*SD*)

| Daily consumption [g per animal]<br>Dzienne zużycie [g na mysz/szczur] | Rat<br>Szczur | Mice<br>Mysz |
|---|---|---|
| | 0.31 ±0.05 | 0.20 ±0.02 |

Table 2. Limits of quantification (LOQ) of the ICP-OES method ($n$ = 5)

Tabela 2. Granice oznaczalności metali (LOQ) metodą ICP-OES ($n$ = 5)

| LOQ [ppm] | Cd | Cu | Fe | Pb | Co | As | Hg |
|---|---|---|---|---|---|---|---|
| | < 0.600 | < 0.869 | < 0.575 | < 1.93 | < 0.614 | < 5.64 | < 0.301 |

Table 3. Heavy metal content in the samples prepared with the migration test method (Method A), $n$ = 3, mean ±*SD*

Tabela 3. Zawartość metali ciężkich w próbkach przygotowanych metodą badania migracji (metoda A), $n$ = 3, średnia ±*SD*

| Enrichment<br>Wzbogacenie | Heavy metal content – Zawartość metali ciężkich [ppm] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cd | Cu | Fe | Pb | Co | As | Hg |
| W1 | < LOQ | < LOQ | < LQ | < LOQ | < LOQ | < LOQ | < LOQ |
| W2 | < LOQ | < LOQ | < LQ | < LOQ | < LOQ | < LOQ | < LOQ |
| W3 | < LOQ | < LOQ | 26.0 ±7.5 | < LOQ | < LOQ | < LOQ | < LOQ |
| W4 | < LOQ | < LOQ | 55.9 ±6.2 | < LOQ | < LOQ | < LOQ | < LOQ |
| W5 | < LOQ | < LOQ | 101.9 ±41.9 | < LOQ | < LOQ | < LOQ | < LOQ |
| W6 | < LOQ | < LOQ | 33.4 ±9.6 | < LOQ | < LOQ | < LOQ | < LOQ |
| W7 | < LOQ | < LOQ | 64.5 ±7.3 | < LOQ | < LOQ | < LOQ | < LOQ |

The obtained values show that only two elements (Cu and Fe) out of seven were at a level higher than the determined limit of quantification using the applied method. The remaining five elements (Cd, Pb, Co, As and Hg) were below LOQ. It is worth noting that the obtained values statistically differ significantly depending on the method used for

Table 4. Heavy metal content in samples prepared by mineralisation method (Method B), $n$ = 3, mean ±*SD*

Tabela 4. Zawartość metali ciężkich w próbkach przygotowanych metodą mineralizacji (metoda B), $n$ = 3, średnia ±*SD*

| Enrichment Wzbogacenie | Heavy metal content – Zawartość metali ciężkich [ppm] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cd | Cu | Fe | Pb | Co | As | Hg |
| W1 | < LOQ | < LOQ | 63.7 ±19.9 | < LOQ | < LOQ | < LOQ | < LOQ |
| W2 | < LOQ | < LOQ | 104.3 ±28.9 | < LOQ | < LOQ | < LOQ | < LOQ |
| W3 | < LOQ | 69.1 ±6.4 | 320.2 ±36.5 | < LOQ | < LOQ | < LOQ | < LOQ |
| W4 | < LOQ | < LOQ | 353.0 ±58.9 | < LOQ | < LOQ | < LOQ | < LOQ |
| W5 | < LOQ | < LOQ | 427.4 ±24.7 | < LOQ | < LOQ | < LOQ | < LOQ |
| W6 | < LOQ | < LOQ | 420.9 ±72.7 | < LOQ | < LOQ | < LOQ | < LOQ |
| W7 | < LOQ | 29.2 ±3.6 | 499.8 ±4.7 | < LOQ | < LOQ | < LOQ | < LOQ |

the preparation of samples. Higher iron and copper contents were obtained for samples which were prepared with wet mineralisation before the analysis. This may be due to the fact that in this method the samples were more dissolved, which resulted in lower filtering losses before ICP-OES analysis.

It is important that the lowest iron content was registered for commercial enrichments not being cardboard elements: for wooden blocs (W1): below LOQ and 63.7 $\mu g \cdot g^{-1}$ of Fe and for cotton cocoons (W2): below LOQ and 104 $\mu g \cdot g^{-1}$ of Fe respectively for A and B method. Among cardboard enrichments, the lowest iron content was found in commercial cardboard tube, which had 26 and 320 $\mu g \cdot g^{-1}$ of Fe respectively for A and B method. "Home-made" cardboard enrichments contained from two to four times more $\mu g \cdot g^{-1}$ of Fe compared to commercial enrichments.

Knowing the daily consumption of cardboard enrichments, the theoretical daily dose of iron and copper took by rodents was calculated. The results are presented in Table 5.

The feeding standards for rodents specify that the amount of iron contained in 1 kg of feed should not exceed 150–250 mg. Assuming that a mouse weighing about 20 g eats 3 g of feed a day and a rat weighing 220 g eats about 14 g of feed, we calculated that a mouse should take 450–750 µg of Fe daily and a rat 2,100–3,500 µg of Fe daily. Since the values shown in Table 5 are well below the established feeding standards for rodents, we can assume that the "theoretical" iron intake from rodent enrichment is low, safe and has no effect on the balanced diet of the animals.

Similar analyses have been carried out for copper, which should not exceed 25 $mg \cdot kg^{-1}$ daily. This means that a mouse can ingest up to 75 µg of Cu daily and a rat can ingest up to 350 µg of Cu. Comparing the data for copper in Table 5 with the feeding standards, it can be seen that they are well below the acceptable limits, which guarantees that the "theoretical" amount of copper consumed with enrichment should not affect the health of rodents. This is the same conclusion as for the iron content.

Table 5. Daily intake of iron and copper from cardboard enrichment ($n$ = 3, mean ±*SD*)
Tabela 5. Dzienny pobór żelaza i miedzi ze wzbogaceń kartonowych ($n$ = 3, średnia ±*SD*)

| Metal | Test animal Testowane zwierzę | Daily intake – Dzienna dawka [µg] | | | | |
|---|---|---|---|---|---|---|
| | | W3 | W4 | W5 | W6 | W7 |
| | | Method A – metoda A | | | | |
| Fe | mice mysz | 5.2 ±1.5 | 11.2 ±1.2 | 20.4 ±8.4 | 6.7 ±1.9 | 12.9 ±1.5 |
| | rat | 8.2 ±2.3 | 17.5 ±1.9 | 31.9 ±13.1 | 10.5 ±3.0 | 20.2 ±2.3 |
| | | Method B – metoda B | | | | |
| Fe | mice mysz | 64.0 ±7.3 | 70.6 ±11.0 | 85.5 ±4.9 | 84.2 ±14.5 | 100.0 ±0.9 |
| | rat szczur | 100.4 ±1.4 | 110.7 ±18.2 | 134.1 ±7.7 | 132.0 ±22.8 | 156.8 ±1.5 |
| Cu | mice mysz | 5.8 ±0.7 | – | – | – | 21.7 ±1.7 |
| | rat szczur | 9.2 ±1.1 | – | – | – | 13.8 ±1.1 |

## CONCLUSIONS

The results of the analysis of environmental enrichment samples from households and commercially available enrichments show that they do not contain toxic heavy metals such as Cd, Pb, Co, As and Hg. Low content of copper was observed in the “home-made” tubes and iron for all analysed samples. The amount of copper and iron differed depending on the method of sample preparation. The lower content of metals was observed in the samples mineralised by the migration method, which most probably is associated with a weaker dissolution of cardboard samples. For both elements, a very low level of their content in 1 g of enrichment was determined, which does not exceed the nutritious standards for the microelements.

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## ZAWARTOŚĆ METALI CIĘŻKICH WE WZBOGACENIACH ŚRODOWISKOWYCH DLA MYSZY I SZCZURÓW LABORATORYJNYCH

**Streszczenie.** Dobrostan zwierząt jest definiowany jako stan, w którym zwierzęta bytujące w określonym otoczeniu potrafią radzić sobie z presją tego środowiska życia, czyli mają komfort behawioralny i fizyczny. Aby poprawić zwierzętom laboratoryjnym stan ich bytowania, stosowany jest szeroki wachlarz elementów wzbogacających środowisko. Należą do nich profesjonalnie przygotowane elementy z poliwęglanu (domki, rurki), bawełny, drewna osikowego i celulozy oraz przygotowane „domowym sposobem" rurki celulozowe pochodzenia gospodarczego. Wzbogacenie profesjonalne cechuje wysoki standard wykonania oraz gwarancja bezpieczeństwa stosowania, co w przypadku „domowych" rurek celulozowych może być trudne do spełnienia, w szczególności gdy zawierają one barwniki, tusze drukarskie i resztki klei. W przeprowadzonych badaniach analizowano zawartość metali ciężkich w celulozowych rurkach pochodzenia gospodarczego, stosując dwie metody mineralizacji materiału badanego oraz metodę ICP-OES (optyczna spektrometria emisyjna z plazmą wzbudzoną indukcyjnie). Jako materiał porównawczy posłużyły profesjonalne materiały, tj.: tunele z drewna osikowego, rurki celulozowe oraz kokony bawełniane. Wyniki analizy wykazały, że oba rodzaje wzbogacania środowiska naturalnego („domowej roboty" i „handlowej") nie zawierają toksycznych metali ciężkich. Jedynymi oznaczonymi w badaniu zanieczyszczeniami były żelazo i miedź, których zawartość w analizowanym materiale różniła się w zależności od sposobu przygotowania próbki. Zawartość tych metali była znacznie mniejsza od norm żywieniowych dla gryzoni.

**Słowa kluczowe:** metale ciężkie, wzbogacenia, zwierzęta laboratoryjne, tutki tekturowe, zanieczyszczenia