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Element Determination in Hair by ICP-MS Method: Selection of a Suitable Sample Washing Procedure

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Abstract

Human hair is a biological sample that is, unlike blood, noninvasively collected and can be used in the assessment of element intake. Before analysis, hair samples need to be washed to eliminate external contamination, for which no standard procedure exists. The present study evaluated the efficiency of different processes for washing hair samples (by non-ionic detergent, acids, solvents, and their mixtures), including ultrasonication before ICP-MS element (As, Ca, Cd, Cu, Fe, Hg, Mg, Mn, Mo, Pb, Se, and Zn) analysis. All tested washing procedures using detergent or solvents were satisfactory, while nitric and hydrochloric acid solutions yielded to "released" and lost elements due to damaged hair (visible discolouration and hair impairment). The application of ultrasonication improved washing efficiency up to 10 %, depending on the tested element and washing procedure.

Keywords

Human hair sample, washing procedure efficiency, metal analysis, endogenous and exogenous elements

1 Introduction

Human hair is the filamentous appendage of the skin. Its usage as an alternative biological specimen for various analyses has been studied for decades as it can be easily collected and requires no specific conditions for storage. However, it is a structurally complicated tissue and the most sophisticated biological composite material that should be adequately prepared for particular chemical analysis.[1–3]

Hair grows from the hair follicle bulbs embedded in the inner skin layers (so-called dermis) where the germination centre is formed by matrix cells. Oxygen and nutrients from the blood fuel the growth of constantly dividing matrix cells, pushing up new cells from the blood supply and resulting in gradual cell death. The process is called keratinization, due to formation of the hard, protective protein keratin. The proteins are synthesized in the keratogenous zone of the hair follicle and move upward from the hair bulb to form layers (cuticle, cortex, and medulla) of the hair shaft, the part of hair that can be seen above the scalp. Considering its morphology and chemistry, hair is defined as being formed by the follicle and the shaft, whereas the formation of the material in the follicle bulb and growing from it through the skin pore is necessary for the organisation of the protein into a fibre. Hair grows approximately 1 cm *per* month, and the growth cycle can be divided into three phases: the anagen phase (growth phase), the catagen phase (transition phase), and the telogen phase (resting phase). About 90 % of hair follicles are in the active anagen phase, and 10 % in the inactive telogen phase. Hair consists of ~80 % protein (mainly as α-keratin chains), 15 % water, and a low proportion of lipids and trace elements. Keratin is rich with sulfhydryl groups to which metal ions have a high binding affinity, thus resulting in the incorporation of metals into hair.[1–6]

Elements in hair can be of either endogenous or exogenous origin. Endogenous elements incorporate in hair from the blood supply into keratinous matrix as hair grows, and they give us information about metal exposure of a person that can occur by industrial emission, environmental contamination (including cigarette smoking), occupational exposure, volcanic gasses, and emissions by coal-fired power plants.[4–8] Exogenous elements are bound to hair surface and reflect possible contamination due to external factors, such as dust particles, water used for washing hair, and cosmetic hair treatments, sweat, collection, and storage of hair samples.[1,4,5] The exogenous elements are the main limitation to using hair as an acceptable biological sample as they may affect analytical results and lead to misinterpretation of the results used for the assessment of

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element exposure and intake, health risk of toxic element exposure or metal intoxication. Having all this in mind, exogenous elements must be removed before analysis by adequate hair sample washing procedures while the hair structure should be preserved so that endogenous elements are unspoiled.[1,7,9]

The most commonly used biological samples to assess current metal exposure in human biomonitoring (HBM) is peripheral venous blood, urine, and faeces. The two latter types of samples, as opposed to blood, are noninvasively collected samples. Hair is also a noninvasively collected biological sample that can be used in HBM as a more ethically appropriate and easy-to-collect sample that requires no special physicochemical conditions for its transport and storage. Another advantage of its use is that elements in hair, especially toxic elements, are present at least at a ten times higher concentration than those in the blood. Furthermore, element levels in hair can give us information about long-term exposure as human hair grows 1 cm *per* month, unlike blood, which reflects only recent exposure due to the fact that most of the elements bound to red blood cells live in circulation for about 100 to 120 days. Despite these advantages, hair as a sole biological sample, is not fully accepted as a relevant sample in HBM for most toxic metals (except for Hg and As).[1,5–8] This is because mechanisms of element incorporation in hair are still not fully understood, and there is a lack of sufficient information about the relationship between element concentrations in hair, blood, urine, faeces, and tissue. There is also an insufficiency of reference ranges assessed with taking into account age, sex, and cultural habits and traditions.[1,6] In addition, there is no standardized procedure for washing hair, which is the first and most important step before element analysis to bring out accurate measurements and comparable results. Therefore, further studies are needed, firstly, to establish a standard washing protocol, and secondly, reference ranges for elements in human hair.

The International Atomic Energy Agency (IAEA)[10] recommended a washing procedure to remove external contamination from hair that involves washing 5–10 min with non-polar solvent acetone, followed by washings in water as a polar solvent, and again with acetone (with or without ultrasonication). They also suggested using a non-ionic detergent instead of one step with water. However, many studies do not apply this procedure, and have applied various washing procedures, *e.g.*, only water and/or detergent,[11–14] different organic solvents or mixture of solvents,[15–18] and acidic solutions at low pH.[19,20]

To the best of our knowledge, this is the first study where washing procedures presented in literature were tested alongside one another. The present study aimed to examine the washing procedures for removing external contamination from hair samples before element (As, Ca, Cd, Cu, Fe, Hg, Mg, Mn, Mo, Pb, Se, and Zn) analysis by ICP-MS method.

2 Experimental

2.1 Preparation of hair samples homogenates

Scalp hair samples of unknown persons (N = 2) of 20–30 cm length and about 2 cm thick were collected in a women's hairdressing salon (hereinafter referred to as "sample – 1" and "sample – 2"). "Sample – 1" was naturally brown hair, while "sample – 2" was grey hair. In the laboratory, the whole length of each hair sample was cut into small pieces, and mixed to obtain a homogeneous sample. From these two homogeneous samples, 26 subsamples were taken (≈ 0.100 g) to assess the washing efficiency of different solutions used for washing hair before element analysis.

2.2 Washing hair samples

For this study, samples prepared after homogenization were washed using three or four steps with 13 different washing procedures, as shown in Fig. 1. Hair samples (≈ 0.100 g) were weighed into empty quartz tubes and washed. In the first step of washing, an organic solvent (acetone, methanol, ethanol, a mixture of ethyl acetate and acetone (3 : 1)) or acid (nitric (6.7 % *v/v*) and hydrochloric (2 % *v/v*)) was used. Then followed rinsing 2–3 times with ultrapure water (more if necessary), and at the end, again with a solvent or diluted acid. In the four-step hair sample washing procedure, a non-ionic detergent (TX-100) was used in the second step, before rinsing with water. All solutions (except water) were in contact with hair for about 10 min with occasional vortex mixing. The application of an ultrasonic bath during the first step was also tested. All of the samples were dried at 75 °C for 2–3 h and weighed after cooling.

2.3 Element analysis

Washed, dried, and weighed hair samples were digested with 2 ml of sub-boiled concentrated nitric acid and ultrapure water (1 : 1) in a microwave digestion system UltraCLAVE IV (Milestone, Italy) according to the manufacturer's instructions (Table 1). The sub-boiling distillation duoPUR system (Milestone, Italy) was used for purified nitric acid (65 % *p.a.*, Merck, Germany). After digestion, the sample was adjusted with ultrapure water (GenPure, TKA System GmbH, Germany) to 6 g, and samples were stored at +4 °C before element analysis. The 700 µl of digested hair samples were diluted to a total volume of 3 ml by 1 % (*v/v*) HNO_3 with 3 $\mu g\,l^{-1}$ of internal standard (Ge, Rh, Tb, Lu, and Ir), which were used to correct variability between the calibration standards and the samples. Element concentrations (As, Ca, Cd, Cu, Fe, Hg, Mg, Mn, Mo, Pb, Se, and Zn) were determined by an inductively coupled plasma – mass spectrometer (ICP-MS) Agilent 7500cx (Agilent Technologies, Japan) according to conditions shown in Table 2. All steps of sample preparation and analysis were done in a laboratory with standard heating, ventilation, and air conditioning (HVAC) system combined with high-efficiency particulate air (HEPA) filters. Calibration curve standards prepared in 1 % HNO_3 were used for quantification, while limit of detection (LOD) and quantification (LOQ) were calculated as mean plus three times (LOD) and ten times (LOQ) of the blanks' standard deviation. The LOD were 0.002–0.006 µg kg^{-1} (LOQ 0.006–0.02 µg kg^{-1}) for Cd, As, Mo, Pb, and Se, 0.02–0.03 µg kg^{-1} (LOQ 0.06–0.09 µg kg^{-1}) for Hg and Mn, 0.3–0.9 µg kg^{-1} (LOQ 0.9–3 µg kg^{-1}) for Cu and Mg, and 1–10 µg kg^{-1} (LOQ 4–10 µg kg^{-1}) for Ca, Fe, and Zn. Commercially available reference materials: human hair IAEA-086 (IAEA, Austria), human hair NIES No. 13 (NIES, Japan), and bovine liver NIST SRM 1577a were used for checking the analytical accuracy of the measurement. In this study, 12 elements were analysed, and all were covered by at least one of the selected reference materials. Recovery data were satisfactory, from 91–110 % for all measured elements except for Fe in IAEA-086 (114 %), and Mn in NIES No. 13 (85 %).

Table 1 – Temperature program for hair samples digestion by UltraCLAVE IV microwave digestion system (Milestone, Italy)
Tablica 1 – Temperaturni program za razaranje uzoraka kose u uređaju za visokotlačno mikrovalno razaranje UltraCLAVE IV

	t / min:s	E / W	T / °C	p / bar
1.	5	1000	80	100
2.	10	500	130	100
3.	5	1000	180	120
4.	7 : 30	1000	220	130
5.	20	1000	220	130
6.	40	0	20	10

Table 2 – ICP-MS operating conditions
Tablica 2 – Uvjeti mjerenja na uređaju ICP-MS

Parameter			
RF Power	1550 W		
RF matching	1.72 V		
Sampling depth	7.5 mm		
Torch-H	0.3 mm		
Torch-V	−0.4 mm		
Nebulizer pump	0.08 rps		
Plasma gas flow rate	15 l min^{-1}		
Make up flow rate	0.17 l min^{-1}		
Carrier gas flow rate	1.0 l min^{-1}		
Nebulizer	MicroMist (quartz)		
Spray chamber	Scott type (quartz), cooled at 2 °C		
Ni cones, diameter	1 mm *Sampling cone;* 0.4 mm *Skimmer cone*		
Doubly-charged ions and oxides limits	$^{140}Ce^{2+}/^{140}Ce^{+} < 1.2$ %; $^{140}Ce^{16}O^{+}/^{140}Ce^{+} < 1.3$ %		
Collision/reaction gas	**No gas**	**He**	**H_2**
Collision/ reaction gas flow rate	/	4.1 ml min^{-1}	4.5 ml min^{-1}
Extract lens 1 voltage	0 V	0.5 V	0 V
Extract lens 2 voltage	−140 V	−140 V	−135 V
Measured isotopes	^{202}Hg	^{24}Mg, ^{43}Ca, ^{55}Mn, ^{56}Fe, ^{63}Cu, ^{68}Zn, ^{75}As, ^{95}Mo, ^{111}Cd, ^{208}Pb	^{78}Se

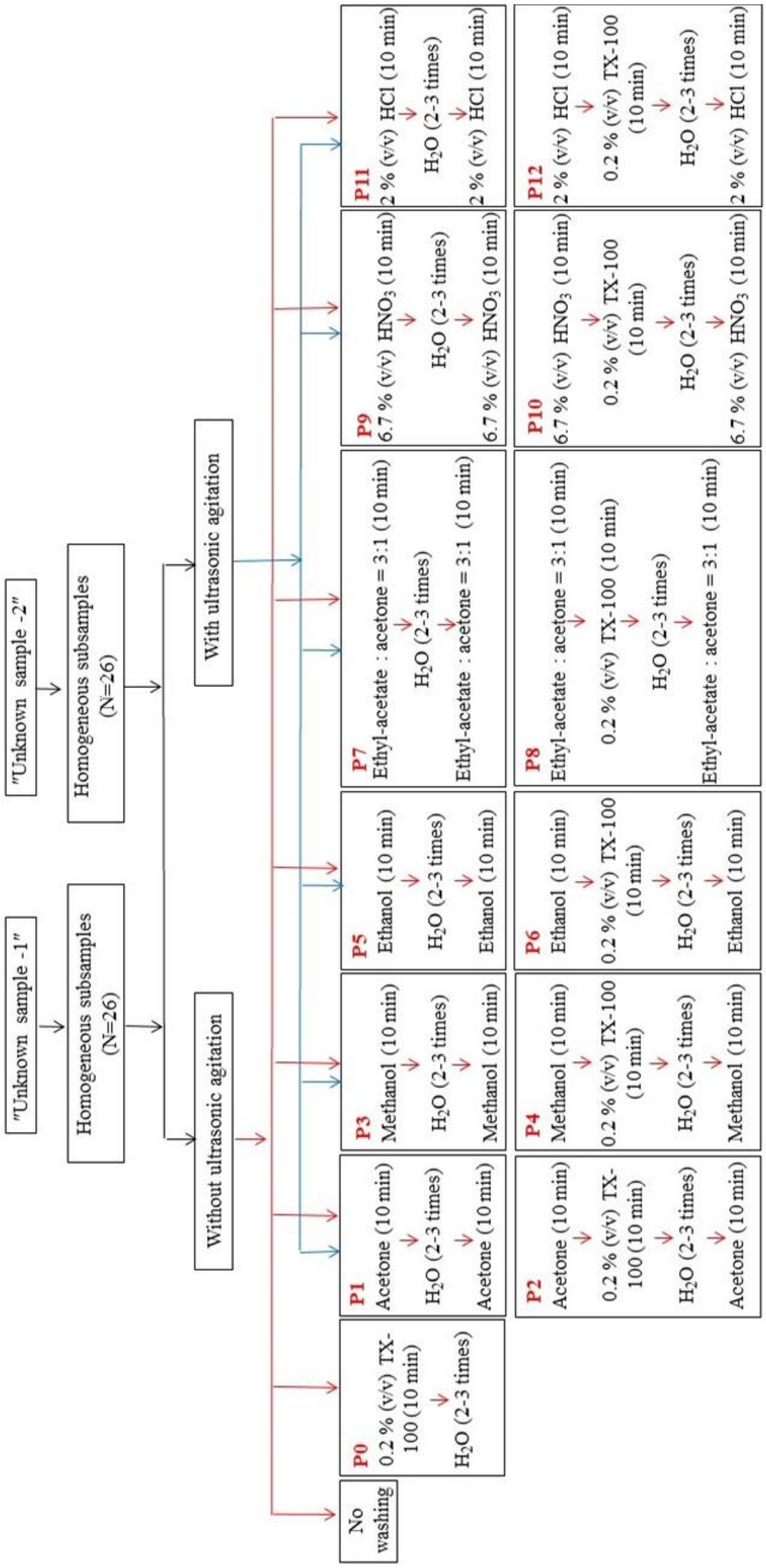

Fig. 1 – Schematic illustration of tested washing procedures used for washing human hair samples before element analysis
Slika 1 – Shematski prikaz ispitivanih načina pranja kose za analizu elemenata

2.4 Data analysis

Data were presented as the mean ± standard deviation (SD). The *paired two-tailed* Student's *t-test* was used to test the differences between the tested washing procedure and IAEA recommended procedure P2. Statistical significance was set at 5 % ($p < 0.05$). Washing efficiency (% washed) was calculated as the percentage of the difference between the ratio of element concentrations before or after washing, and unwashed value.[19,21] Statistical analysis and graphical illustrations were performed using Microsoft Excel Office Professional 2016 (Microsoft, USA).

3 Results and discussion

There is still no generally accepted standard procedure how to wash hair samples before element analysis, capable of both eliminating external contaminations, and preserving internal elemental content. It is difficult to prove to what extent a particular washing procedure removes surface contaminants or is abrasive and causes damage to the hair sample, leaching the elements or even diffusing them into the hair.[4] As mentioned previously, most studies use only water and/or detergent, organic solvents or solvent mixtures. The washing procedures included three or more washing steps, such as: non-ionic detergent – water – acetone,[22,23] water – detergent – water – methanol,[15] detergent – water – ethanol,[18] acetone – water – acetone,[24] and acetone – detergent – water – acetone[25], which is a method of washing recommended by the IAEA.[10] Instead of an organic solvent, several studies used acids to remove external contamination from hair.[19–20]

In this study, we evaluated the washing efficiency of different washing procedures reported in the literature for washing hair samples before element analysis. Through the determination of element concentrations in unwashed hair samples, we obtained information on the primary element levels in the hair, which represent the total concentration of the elements, including possible contamination.

Figs. 2 and 3 show the concentrations of 12 elements in two hair samples washed with 13 different washing procedures. The two hair samples we used for testing these washing procedures were visibly mutually different: "sample – 1" was naturally brown hair, while "sample – 2" was grey hair. The element levels in the hair were also different between the samples. The brown hair sample had higher concentrations of Ca, Mg, Zn, and Hg, and lower concentrations of Mn and Pb, while the levels of other elements were the same in both samples. The concentration of elements in hair may be associated with age,[26,27] although several studies failed to find such association.[28,29] Due to contradictory results in the literature, studies using element levels in human hair need to take into account the factors that may have an impact on element levels, such as natural hair colour, personal dietary habits, metabolic processes, sex, race, as well as type of element exposure, that is, whether it is everyday environmental element exposure (by food, water or cigarette smoking) or specific occupational exposure to particular metal/s.

Different elements have different properties and binding affinity in hair, and the selection of an adequate sample washing procedure for multi-element analysis are a challenge. We found differences between P2, IAEA protocol, which we set as a suitable general procedure of hair sample washing for multi-elemental analyses, and procedures in which nitric or hydrochloric acid solutions were used (P9–P12) for all analysed elements except Se in both hair samples, and As and Mg in "sample – 2". Regarding washing procedures using detergents and different solvents and their mixtures (P0–P8) *vs*. P2, we found differences for As (P0), Ca (P1), Cu (P3, P4, and P5), Fe and Mg (P0 and P1), Mo (P5, P7, and P8) and Zn (P0, P4, P6, P7, P8) in "sample – 1" and for Cd and Pb (P0), and Fe in P4 and P6 in "sample – 2". The results show that the washing efficiency depends on the selected solution (organic solvent or acid), but also the analysed element and the hair characteristics. We also calculated the washing efficiency for all tested procedures. When only non-ionic detergent, 0.2 % TX-100 (procedure P0) was used, the washing efficiency was in the range of 1–5 % for Cu, Mo, Pb, and Zn, 13–20 % for Fe, Mg, and Mn in both samples. The washing efficiency in P0 for Ca, Hg, and Se in the "sample – 1" ranged 2–11 %, and 19–23 % for As and Cd, while in the "sample – 2" this range was 0–6 % for As, Cd, and Se, and 14–19 % for Ca and Hg. Washing procedures, when the step with TX-100 was added to washing with organic solvents (P2, P4, P6, P8), had the same or an up to 2 % higher washing efficiency, except for As (6–13 %), Cd, Cu, Mg, Mn, and Zn (up to 5 %) in "sample – 1" and Cd, Mg, and Pb in "sample – 2" (4–11 %) in some of them. Organic solvents such as ethanol, methanol, acetone, or ethyl-acetate partially removed grease, dust, and organic impurities, while non-ionic detergent further enhanced hair sample washing. A study conducted in 75 men (age 25–35) showed that external contamination was not removed in hair washed with distilled water, while ethanol and acetone eliminated organic contamination (primarily oils, lacquers and particulate matter). It was concluded that the best effect of washing hair was accomplished by applying a washing procedure that included non-ionic detergent and acetone together, because non-ionic detergent removes grease and dust from the hair, while the organic solvent removes organic components.[21] The IAEA hair sample washing procedure[10] was proposed as a standard. The washing method comprises two 10 min washing steps with acetone, a good and extremely dry degreaser.

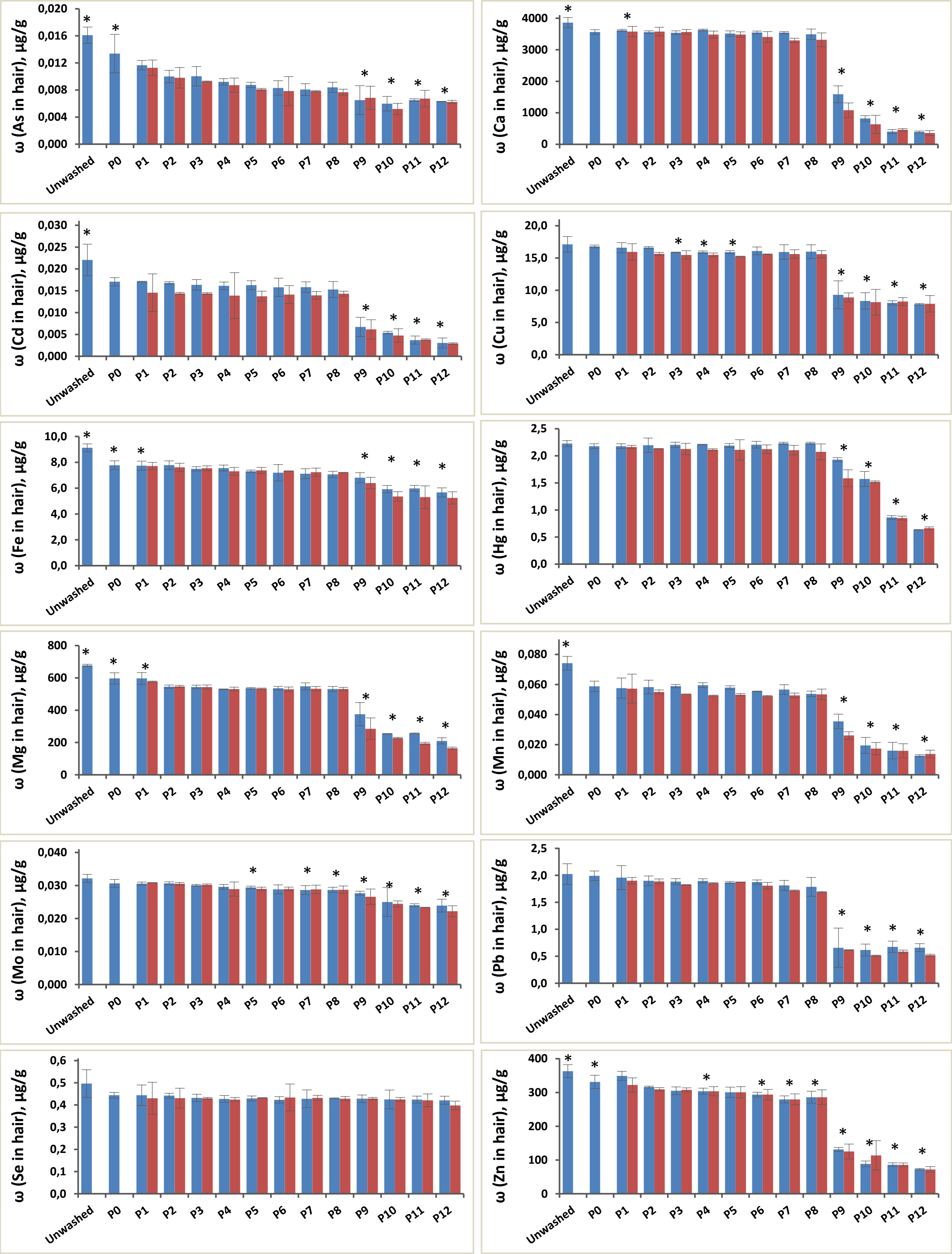

Fig. 2 – Element concentration (mean ± SD) in homogenates of human hair "sample – 1" washed with different washing procedures (P0–P12 described in Fig. 1) with ultrasonic agitation (indicated in red) and without ultrasonic agitation (indicated in blue). *Statistically significant differences (at $p < 0.05$) between the tested washing procedure *vs.* procedure P2 tested by Student's *t*-test.

Slika 2 – Koncentracija elemenata (srednja vrijednost ± SD) u homogenatima uzorka ljudske kose – 1 oprane različitim postupcima pranja (P0 – P12 opisano na slici 1) uz (prikazano crveno) primjenu i bez primjene ultrazvučnih valova (prikazano plavo). *Statistički značajna razlika ($p < 0{,}05$) između testiranog postupka pranja *vs.* postupak P2 testirana Studentovim *t-testom*.

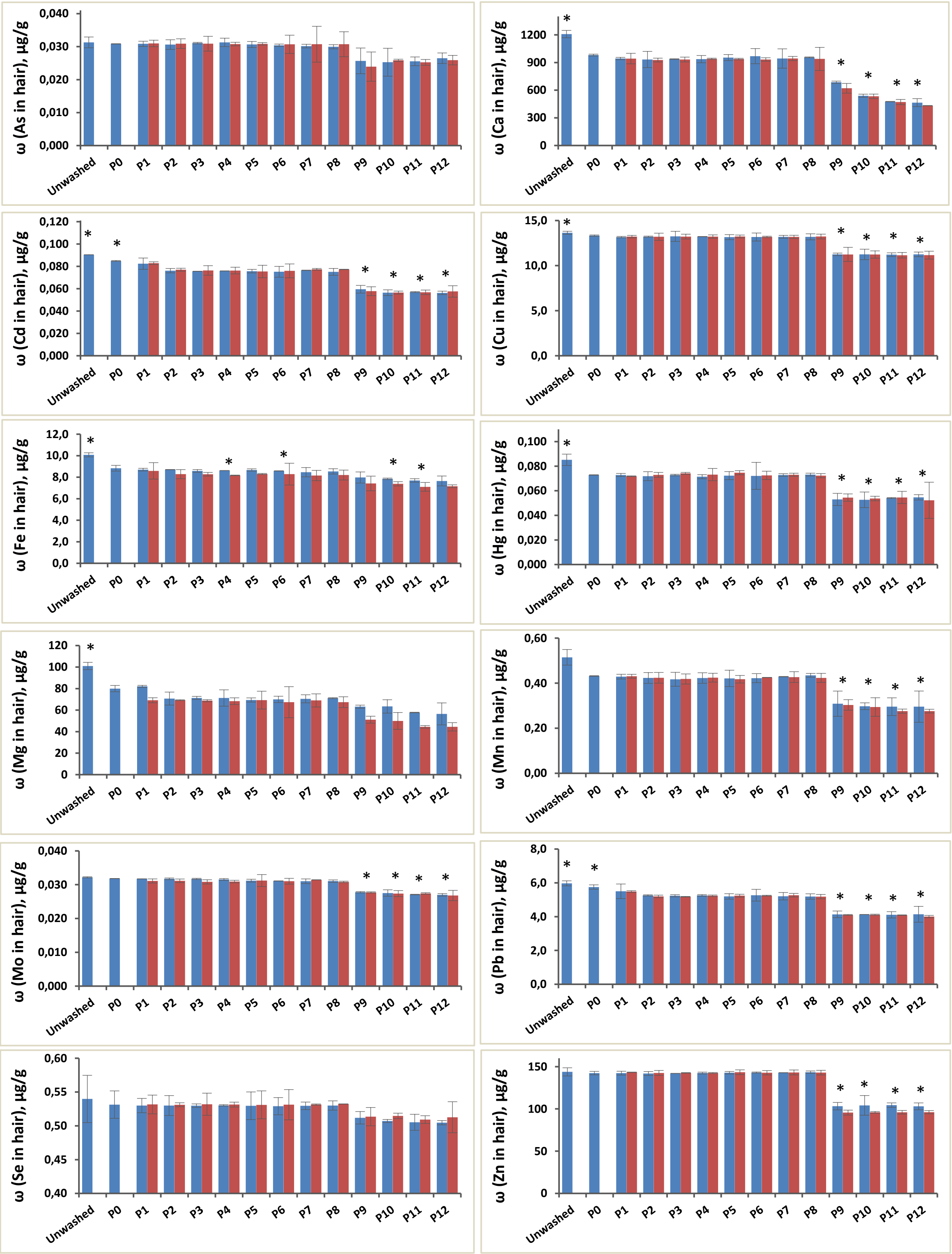

Fig. 3 – Element concentration (mean ± SD) in homogenates of human hair "Sample – 2 washed with different washing procedures (P0–P12 described in Fig. 1) with ultrasonic agitation (indicated in red) and without ultrasonic agitation (indicated in blue). *Statistically significant differences (at $p < 0.05$) between the tested washing procedure *vs.* procedure P2 tested by Student's *t-test*.

Slika 3 – Koncentracija elemenata (srednja vrijednost ± SD) u homogenatima uzorka ljudske kose – 2 oprane različitim postupcima pranja (P0 – P12 opisano na slici 1) uz (prikazano crveno) primjenu i bez primjene ultrazvučnih valova (prikazano plavo). *Statistički značajna razlika ($p < 0{,}05$) između testiranog postupka pranja *vs.* postupak P2 testirana Studentovim *t-testom*.

However, it was not generally accepted and used in studies, especially not in those where a large number of elements were measured.[11,15,18] The argument was that, although acetone effectively removed solid particles from the hair surface, it also produced microscopic damage to the hair, observed by a scanning electron microscopy-energy dispersive X-ray (SEM-EDX). Instead of acetone, ultrapure water as washing reagent was used by *Raposo et al.*[30], regardless of the fact that endogenous and exogenous elements could not be differentiated. However, *Mikasa's et al.*[31] data showed that there was no loss of elements when the washing step with acetone was included. Another study showed that acetone significantly reduced Cd level by the same percentage regardless of high or low element content in hair samples.[32] In many studies, hair was washed with different procedures, and we tested some of these procedures. The results showed that all of the tested washing procedures, including the use of non-ionic detergent, organic solvents, and water in different combinations (P1–P8), almost equally removed a certain element from the hair surface, and the addition of detergent improved (up to 2 %) the washing efficiency. An exception to this conclusion are procedures where diluted acid solutions were used to remove the contamination from hair (P9–P12). When 6.7 % v/v nitric acid (P9 and P10) was used for washing hair, the washing efficiency ranged from 46–79 % for all measured elements except for Fe, Hg, Mo, and Se (13–41 %) in "sample – 1". For "sample – 2", this range was 28–55 % for all elements except for As, Cu, Fe, and Mo (14–22 %), and Se, where the washing efficiency was less than 10 %. During these procedures, hair damage could also be noticed visually as a discolouration and hair impairment, already at the first step of the washing procedures. The washing efficiency in the procedures where 2 % (*v*/*v*) hydrochloric acid solution was used (P11 and P12) was 2–30 % higher or very similar to the results obtained with nitric acid, except for Hg, where the washing efficiency was 40 % higher.

In a recently published study (N = 3), *Verrey et al.*[19] tested three washing protocols for human hair (non-ionic detergent – nitric acid, IAEA procedure, and non-ionic detergent – nitric acid – hydrochloric acid) for 23 elements, and proposed a new hair sample washing procedure, which included 0.5 % (*v*/*v*) TX-100, 6.7 % nitric and 2 % (*v*/*v*) hydrochloric acid at low pH values in an ultrasonic bath during 5 min at each step. The authors explained their use of acid for washing hair samples with better washing efficiency than usual procedures with acetone and water, and compared their results to those obtained for washing metals from contaminated sediments. However, our results showed that acid is not suitable as a washing solution, because it leads to hair damage, and we cannot be sure that only exogenous elements are removed from the hair surface or if endogenous elements are also removed due to the destruction of the hair structure.

In this study, we also assessed the application of ultrasonication on washing efficiency so that all tested washing procedures were repeated with ultrasonication performed for 10 min during the first washing step. We found that ultrasonication improved the washing efficiency up to 10 %, depending on the element and washing procedure. The ultrasonication used in surface cleaning was due to the mechanical and physical effects of ultrasounds, which may reduce the use of chemical solvents and improve the speed and cleaning efficiency.

4 Conclusion

The washing step is an essential point in element analysis of human hair, because it is important to remove external contaminations that may have an impact on metal content in hair, although other factors (sex, age, colour, *etc.*) can contribute to data variability. In this study, we evaluated the washing efficiency of different washing procedures used for washing human hair samples before element analysis, because to date, no standard washing procedure has been accepted. The results showed that all of the tested washing procedures were satisfactory in removing exogenous elements from the hair surface. At the same time, the addition of non-ionic detergent improved the washing efficiency up to 2 %. The use of acid (nitric or hydrochloric) for washing hair samples is unacceptable because it causes hair damage and can result in "releasing" elements from the hair. The application of ultrasonic agitation improved the washing efficiency up to 10 %, depending on the element and washing procedure. In conclusion, we recommend the washing procedure that includes acetone for 10 min in an ultrasonic bath, 10 min in non-ionic detergent with occasional mixing, rinsing several times with water, and 10 min in acetone again, as the optimal method of hair sample washing before element analysis, which is in line with the procedure recommended by the International Atomic Energy Agency. It is important to use the same or slightly modified washing procedure for hair samples in order to obtain mutually comparable results for a large range of elements analysed by ICP-MS.

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List of abbreviations and symbols
Popis kratica i simbola

HBM	– human biomonitoring – biološki monitoring ljudi
IAEA	– International Atomic Energy Agency – Međunarodna agencija za atomsku energiju
ICP-MS	– inductively coupled plasma – mass spectrometer – spektrometrija masa uz induktivno spregnutu plazmu
SEM-EDX	– scanning electron microscopy – energy dispersive X-ray – pretražni elektronski mikroskop s energetski disperzivnom rendgenskom spektroskopijom

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SAŽETAK

Određivanje elemenata u kosi metodom ICP-MS: odabir odgovarajuće procedure pranja uzorka

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Ljudska kosa je biološki uzorak koji se, za razliku od krvi, skuplja neinvazivno i može rabiti u procjeni unosa elemenata. Prije analize uzorke kose potrebno je oprati kako bi se odstranila vanjska onečišćenja za što ne postoje standardni postupci. Istraživanjem je procijenjena učinkovitost različitih postupaka pranja uzoraka kose (neionskim detergentom, kiselinama, otapalima i njihovim mješavinama) uključujući primjenu ultrazvuka prije analize elemenata (As, Ca, Cd, Cu, Fe, Hg, Mg, Mn, Mo, Pb, Se, and Zn) metodom ICP-MS. Ispitivani postupci pranja bili su zadovoljavajući, izuzev primjenom dušične i klorovodične kiseline, pri čemu se elementi "otpuštaju" i gube zbog oštećivanja kose (vidljive su promjena boje i pucanje vlasi). Primjena ultrazvuka pospješila je učinkovitost pranja uzoraka do 10 % ovisno o ispitivanom elementu i postupku pranja.

Ključne riječi

Uzorak ljudske kose, učinkovitost postupka pranja, analiza metala, endogeni i egzogeni elementi

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