



Is there a difference in the phthalate exposure between adults with metabolic disorders and healthy ones?

Da li postoji razlika u izloženosti ftalatima između odraslih osoba sa metaboličkim poremećajima i zdravih osoba?

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Abstract

Background/Aim. Phthalates are recognized as endocrine-disrupting compounds and are extensively present in a variety of everyday products. Chronic exposure to phthalates is suspected to be associated with a range of health disorders. The aim of the study was to examine the abundance of phthalate metabolites in the urine samples among adults in the Autonomous Province of Vojvodina, Serbia, and to determine the prevalence of phthalate metabolites in healthy individuals and those with metabolic disorders such as obesity and newly diagnosed type 2 diabetes mellitus (T2DM). **Methods.** For the study purpose, the first morning urine sample of 308 participants was screened for the presence of 10 phthalate metabolites: mono-ethyl phthalate (MEP), mono-(2-ethylhexyl) phthalate (MEHP), mono-n-butyl phthalate (MBP), mono-iso-allyl phthalate (MiAP), mono-n-allyl phthalate (MnAP), mono-cyclohexyl phthalate (MCHP), mono-benzyl phthalate (MBzP), mono-n-octyl phthalate (MOP), mono-n-propyl phthalate (MPP) and mono-methyl phthalate (MMP). **Results.** At least one

phthalate metabolite was detected in the first morning urine sample in 50.32% of the examined population. The most frequently detected phthalate metabolites were MEP and MEHP. Out of all phthalate-positive participants, 38.3% of them had one, 10.7% had two, while 1.3% of participants had three phthalate metabolites in the first morning urine sample. A significant difference ($p < 0.05$) between groups was observed on MEP and MMP frequency, while borderline significant difference ($p < 0.1$) between groups was observed on MEHP and MCHP frequency. **Conclusion.** In the Vojvodina region, both healthy adults and those with metabolic disorders such as obesity and newly diagnosed T2DM are predominantly exposed to di-ethyl phthalate and di-(2-ethylhexyl)phthalate since MEP and MEHP were the most frequently detected phthalate metabolites. Further research is required in order to provide more details of the phthalates influence on the adverse health effects.

Key words: endocrine system diseases; phthalic acid; monoethyl phthalate; risk assessment; serbia.

Apstrakt

Uvod/Cilj. Ftalati predstavljaju grupu jedinjenja za koje je poznato da imaju štetan uticaj na endokrini sistem, a nalaze se u različitim potrošačkim proizvodima. Hronična ekspozicija ftalatima dovodi se u vezu sa nastankom mnogobrojnih oboljenja. Cilj rada bio je da se utvrdi zastupljenost ftalatnih metabolita u uzorcima urina odraslih osoba u Vojvodini, Srbija, kao i da se utvrdi prevalenca ftalatnih metabolita kod zdravih ispitanika i onih sa metaboličkim poremećajima kao što su gojaznost i novootkriveni dijabetes melitus tip 2 (T2DM). **Metode.** Za

ispitivanje je korišćen prvi jutarnji uzorak urina 308 ispitanika koji je analiziran na prisustvo 10 ftalatnih metabolita: mono-etil ftalata (MEP), mono-2-etilheksil ftalata (MEHP), mono-n-butil ftalata (MBP), mono-izo-amil-ftalata (MiAP), mono-n-amil ftalata (MnAP), mono-cikloheksil ftalata (MCHP), mono-benzil ftalata (MBzP), mono-n-oktil ftalata (MOP), mono-n-propil ftalata (MPP) i mono-metil ftalata (MMP). **Rezultati.** Kod 50,32% ispitivane populacije u uzorku urina detektovan je najmanje jedan ftalatni metabolit. Najzastupljeniji ftalatni metaboliti bili su MEP i MEHP. Među ispitanicima pozitivnim na prisustvo ftalatnih metabolita, 38,3% ispitanika imalo je detektovan jedan,

10,7% imalo je dva, a 1,3% ispitanika imalo je prisutna 3 ftalatna metabolita u uzorku jutarnjeg urina. Utvrđeno je postojanje značajne razlike ($p < 0,05$) između grupa u prisustvu MEP i MMP ftalatnih metabolita, kao i granične značajnosti ($p < 0,1$) između grupa u prisustvu MEHP i MHCP ftalatnih metabolita. **Zaključak.** U populaciji Vojvodine, i zdrave osobe i one sa metaboličkim poremećajima, kao što su gojaznost i novootkriveni T2DM, domi-

natno su izložene di-etil ftalatu i di-2-etilheksil ftalatu, s obzirom na to da su najzastupljeniji ftalatni metaboliti bili MEP i MEHP. Neophodna su dalja istraživanja koja će omogućiti bolji uvid u štetan uticaj ftalata na zdravlje.

Ključne reči:
endokrini sistem, bolesti; ftalna kiselina; monoetil ftalat; rizik, procena; srbija.

Introduction

Phthalates represent a large group of omnipresent industrial chemicals, ordinarily used as plasticizers, and can make up to 40%–50% of the polyvinyl chloride plastic product's weight. They are known to act as endocrine-disrupting chemicals (EDCs) ¹. Phthalates can be found in food packaging, furniture, toys, and many other household products, but also in medical devices, such as tubing and intravenous bags. Phthalates are also popular in the cosmetic industry. Since they are not covalently bound to the plastic, phthalates can leach and transfer to the air, food, and water, and thus become inhaled, ingested, or absorbed through the skin ². After being absorbed in the circulation, phthalates are metabolized in two phases: hydrolysis (monoester phthalates are produced) and conjugation. Phthalates are mainly excreted through urine, but they can also be detected in various fluids like blood (serum and plasma), breast milk, saliva, feces, etc. ³.

Monoester phthalates have a fairly short half-life in humans. Despite this fact, numerous scientific evidence implies that phthalate diesters and monoesters can lead to health disorders such as mental retardation, body composition problems, as well as endocrine, pulmonary, and cardiovascular diseases ⁴.

Phthalates can activate different pathways, but nuclear receptors (NR) are recognized as a primary target ⁵. Acting as a partial or complete agonist or antagonist, phthalates can alter NR signaling involved in the regulation of the metabolism and energy homeostasis. The main NRs targeted by phthalates are peroxisome proliferator-activated receptors (PPAR α , γ), androgen receptors, thyroid hormone receptors (TR α , β), pregnane X receptor (PXR), estrogen receptors (ER α , β), and estrogen-related receptors ^{6,7}.

By binding to the different components of the PPARs involved in the regulation of adipose tissue and lipid homeostasis, phthalates affect the fat distribution and alter the lipid status ^{8,9}. Moreover, through the PPAR- γ receptor component, phthalates could induce insulin resistance and impair glucose homeostasis. Besides genetic inheritance and lifestyle, chronic exposure to environmental pollutants, including chronic phthalate exposure, may attribute to the global epidemics of obesity and type 2 diabetes mellitus (T2DM) ¹⁰.

The aim of the study was to examine the presence of phthalate metabolites among adults in the Autonomous Province (AP) of Vojvodina (both healthy ones and those with metabolic disorders) in order to find the most abundant

metabolites. An additional aim was to determine the prevalence of phthalate metabolites in the control group, obese and a group of participants with newly diagnosed T2DM.

Methods

A total of 308 participants aged 18–50 years from the Vojvodina region, Serbia, were enrolled in a cross-sectional study. The participants were divided into 3 groups: 103 in the control [healthy persons with normal body mass index (BMI)], 104 in the obese (BMI > 30 kg/m²), and 101 in the group of patients with newly diagnosed T2DM (fasting plasma glucose value > 7.0 mmol/L), without medical treatment.

Participants with a history of chronic diseases such as dyslipidaemia, autoimmune disease, chronic infections, malignant disease, or those with possible or proven pregnancy or lactation were not involved in the study. Participants treated with any kind of medication that could affect the lipid status or the body composition (such as hypolipidemics, glucocorticoids, oral contraceptives, or immunosuppressive drugs) were not included in the study.

The study participants provided written informed consent, and the study protocol was approved by the Ethics Board of the Faculty of Medicine, University of Novi Sad, Serbia. All subjects who decided to withdraw their informed consent were excluded from the study.

Firstly, all participants were surveyed and asked specific questions about their medical and personal history. Afterward, anthropometric values such as weight, height, waist circumference were taken, and BMI was calculated using the following formula – weight/height² (kg/m²). Waist circumference was measured in the middle of the line joining the anterior superior iliac spine and rib arc.

The first morning urine sample of the volunteers who participated in this study was screened for the presence of 10 phthalate metabolites: mono-ethyl phthalate (MEP), mono-(2-ethylhexyl) phthalate (MEHP), mono-n-butyl phthalate (MBP), mono-iso-allyl phthalate (MiAP), mono-n-allyl phthalate (MnAP), mono-cyclohexyl phthalate (MCHP), mono-benzyl phthalate (MBzP), mono-n-octyl phthalate (MOP), mono-n-propyl phthalate (MPP), and mono-methyl phthalate (MMP).

After enzymatical treatment of collected urine samples, methyl-tert-butyl-ether was used as a solvent for the extraction of phthalate metabolites. The samples were prepared and analyzed by the previously developed method

accurately described by Milošević et al.¹¹. Gas chromatography coupled to mass spectrometric detection (Agilent GC 7890A, 5975C VLMSD) equipped with a fused silica capillary column (30 m, 0.25 mm id. and 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA) was used for the determination of phthalates residues in urine. The limit of detection (LOD) for 10 phthalate metabolites was 0.25 ng/mL.

Separate groups were designed for each phthalate metabolite dividing them between phthalate-free and phthalate positive samples (binary distribution), as the span of positive values was too wide so that standard deviations would exclude valuable patients.

Statistical analysis

The data were analyzed using the Pearson's χ^2 test (in cases of a low number of positive phthalate values coefficient of contingency was used) with the significant results being recorded at $p < 0.05$ and $p < 0.1$. The statistical analyses and graphical representation were done using SPSS 23.0 (SPSS Inc., Chicago, Illinois, USA) and MS Excel Package.

Results

General characteristics of the analyzed population such as male to female ratio, gender, height, age, body weight, waist circumference, and BMI values are shown in Table 1. Statistically significant differences in age, body weight, waist circumference, and BMI were

observed among the studied groups.

Phthalate metabolite abundance is shown in Table 2. The most frequently detected phthalate metabolites were MEP and MEHP, while MnAP was the least represented.

Out of 308 participants, 155 (50.32%) had at least one phthalate metabolite detected in the first morning urine sample.

The abundance of phthalate metabolites in each group is shown in Figure 1.

In the control group, the most abundant phthalate metabolites were MEP and MEHP, while MCHP and MMP were not detected. Analyzing the frequency of all metabolites separately, 26 (25.3%) out of 103 participants in the control group had MEP in their urine, 17 (16.5%) had MEHP, and 4 (3.9%) participants were MBP or MOP exposed. Additionally, MiAP and MPP were detected in two samples in the control group (1.7%), while MnAP and MBzP were above the limit of detection in only one sample (0.9%).

In the group of obese participants, MEP and MEHP were also the most abundant phthalate metabolites, while the presence of MCHP again was not determined. In terms of frequency of detection, MEP was determined in the urine of 30 (28.8%) participants, MEHP was found in 27 (25.9%) obese participants, while 4 (3.9%) of them had MBP in their urine sample. Moreover, only one participant was MiAP or MnAP exposed. Three (2.9%) participants were positive with MBzP presence, and 2 (1.9%) of them had MPP in their urine sample. Only 1 (0.9%) participant was positive with MOP presence, while 6 (5.7%) participants had MMP in the urine sample.

Table 1

General characteristics of the entire cluster

Participants	n	Gender (n)		Age (years)	Height (cm)	Weight (kg)	Waist circumference (cm)	BMI (kg/m ²)
		male	female					
Control	103	51	52	35.91 ± 8.00 ^b	173.37 ± 8.44	69 ± 10.71 ^{a, b}	78.38 ± 7.99 ^{a, b}	22.60 ± 2.07 ^{a, b}
Obese	104	51	53	38.61 ± 8.69 ^c	174.10 ± 9.94	106.36 ± 20.37 ^{a, c}	110.29 ± 14.79 ^{a, c}	35.23 ± 6.74 ^{a, c}
T2DM	101	57	44	44.94 ± 7.38 ^{b, c}	171.56 ± 10.83	91.79 ± 23.21 ^{b, c}	103.01 ± 16.81 ^{b, c}	31.18 ± 7.25 ^{b, c}
Total/ Average	308	108	96	39.75 ± 8.87	173.03 ± 9.82	89.08 ± 24.33	97.21 ± 19.36	29.91 ± 7.88

n – number of volunteers; **T2DM** – type 2 diabetes mellitus; **BMI** – body mass index; **Statistically significant difference ($p < 0.01$): a – between control and obese, b – between control and T2DM; c – between obese and T2DM. All values are expressed as mean ± standard deviation or number (n).**

Table 2

Phthalate metabolites abundance in the examined population

Participants	MEP	MEHP	MBP	MiAP	MnAP	MCHP	MBzP	MOP	MPP	MMP
Control (n = 103)	26 (25.3)	17 (16.5)	4 (3.9)	2 (1.9)	1 (1)	0 (0)	1 (1)	4 (3.9)	2 (1.9)	0 (0)
Obese (n = 104)	30 (28.8)	27 (26)	4 (3.8)	1 (1)	1 (1)	0 (0)	3 (2.9)	1 (1)	2 (1.9)	6 (5.7)
T2DM (n = 101)	13 (12.8)	29 (28.7)	5 (4.9)	1 (1)	0 (0)	3 (2.9)	3 (2.9)	1 (1)	0 (0)	9 (8.9)
Total (n = 308)	69 (22.4)	73 (23.7)	13 (4.2)	4 (1.2)	2 (0.6)	3 (0.97)	7 (2.2)	6 (1.9)	4 (1.2)	15 (4.8)

T2DM – type 2 diabetes mellitus; **MEP** – mono-ethyl phthalate; **MEHP** – mono-(2-ethylhexyl) phthalate; **MBP** – mono-n-butyl phthalate; **MiAP** – mono-iso-allyl phthalate; **MnAP** – mono-n-allyl phthalate; **MCHP** – mono-cyclohexyl phthalate; **MBzP** – mono-benzyl phthalate; **MOP** – mono-n-octyl phthalate; **MPP** – mono-n-propyl phthalate; **MMP** – mono-methyl phthalate. All results are presented as number (percentage).

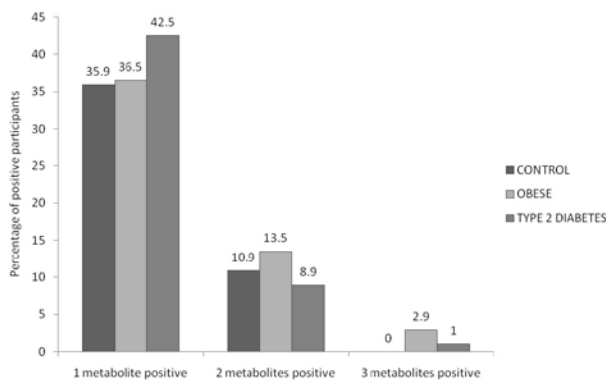


Fig. 1 – Abundance of phthalate metabolites in each individual group.

In the group of participants with T2DM, MEP and MEHP were again the most frequently detected metabolites in the urine, while the presence of MPP and MnAP was not determined. Out of 101 participants with T2DM, 13 (12.8%) had MEP, while 29 (28.7%) of them had MEHP in their urine sample. MBP was detected in the urine of 5 (4.9%) participants, while only one participant was positive with MiAP or MOP presence. Both MCHP and MBzP were detected in the urine of 3 participants, and 9 participants had MMP phthalate metabolite in their urine sample.

The distribution of analyzed metabolites in the phthalate-positive participants is presented in Figure 2. Out of 308 participants, 118 (38.3%) had one phthalate metabolite, 33 (10.7%) of them had two, while 4 (1.3%) participants had three phthalate metabolites in their urine sample. There were no participants with four or more phthalate metabolites detected in their urine samples.

All phthalates were tested for significant differences between the control and obese/T2DM groups of participants. MEP and MMP had p values less than 0.05, while MEHP and MCHP had p values less than 0.1 and, therefore, were considered significant and will be further discussed.

In the examined population of 308 participants, a significant difference was seen in MEP frequency between the control group and T2DM group ($\chi^2 = 5.058$, $df = 1$, $p = 0.025$). Precisely, 26 (25.2%) out of 103 participants in

the control group were MEP positive, exactly double in comparison with the number of exposed T2DM patients (13 out of 101; 12.9%).

Considering the MMP occurrence in urine samples, a significant difference in frequencies was observed between the control group and T2DM group ($\chi^2 = 8.491$, $df = 1$, $p = 0.004$), and the obese group and control group ($\chi^2 = 6.120$, $df = 1$, $p = 0.013$), but one must stress the relative significance of this finding as T2DM group had 9 positive values (9/101, 8.9%), the obese group had 6 (6/104, 5.8%), and the control group had none (0/103, 0%).

MEHP was present in 29 (28.9%) of 101 T2DM participants, in 27 (26%) of 104 in the obese group, and in 17 (16.5%) of 103 in the control group, where a borderline significant difference was observed between the control and T2DM group ($\chi^2 = 3.435$, $df = 1$, $p = 0.064$), and between the obese group and control group ($\chi^2 = 2.765$, $df = 1$, $p = 0.096$).

This metabolite was detected in 3 (3%) of 101 T2DM patients and in none of 103 (0%) participants in the control group with moderate significant difference observed ($\chi^2 = 3.105$, $df = 1$, $p = 0.078$).

Again, among the groups, no statistically significant differences were observed between the characteristics of the MBP, MiAP, MnAP, MBzP, MOP, MPP subgroups.

Discussion

Many chemicals whose presence in nature has been increased after the industrial revolution can act as endocrine disruptors by interfering with endogenous hormonal pathways. Epidemiological studies^{12–14} have shown the link between exposure to these chemicals and the development of common disorders and diseases such as obesity and T2DM. Taking into consideration that the pathogenesis of these disorders depends on the combination of lifestyle habits and genetics, it is lately hypothesized that exposure to endocrine disruptors during or after pregnancy can play a significant role in the onset of some diseases¹⁵. Phthalates are usually found in large quantities in daily products. The number of publications that investigate the positive linkage between

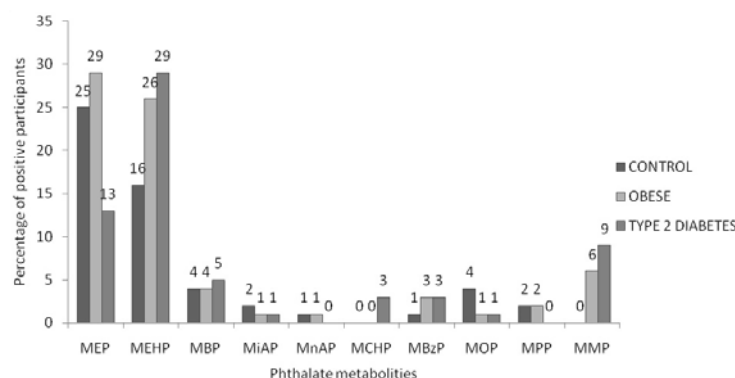


Fig. 2 – Number of phthalate metabolites detected in the urine samples.

MEP – mono-ethyl phthalate; MEHP – mono-(2-ethylhexyl) phthalate; MBP – mono-n-butyl phthalate; MiAP – mono-iso-allyl phthalate; MnAP – mono-n-allyl phthalate; MCHP – mono-cyclohexyl phthalate; MBzP – mono-benzyl phthalate; MOP – mono-n-octyl phthalate; MPP – mono-n-propyl phthalate; MMP – mono-methyl phthalate.

phthalates exposure and adipogenesis and T2DM, some of the largest epidemics of the modern world, increases continuously. Although chemical industry representatives assert that levels of phthalates found in the human body are well below the “safe” concentrations by some regulatory agencies, endocrinologists consider that phthalates exposure, even at low doses, during vulnerable periods, can lead to adverse health effects¹⁶. Although it is estimated that the average level of human exposure to DEHP is around 0.0024 mg/kg/day, much below the current DEHP “No Observed Adverse Effect Level” (NOAEL) by the European Food Safety Authority, the chronic exposure even at low doses could be more harmful than single acute exposure to high dose¹⁷. Natural hormones are active at the pico- to nanomolar range. Hence, phthalates as EDCs might ameliorate hormone homeostasis and cause biological impact at low doses¹⁸. According to literature data^{12, 19}, fetuses, newborns, and adolescents are vulnerable groups and particularly susceptible to phthalate exposure, which is explained by the high levels of cell activity in those age groups.

The obtained results showed that the urine sample of 50.32% of participants was positive for the presence of at least one phthalate metabolite.

The ubiquitous presence of phthalates in human urine samples is documented in the study published by Zota et al.²⁰. Eleven phthalate metabolites were analyzed in the urine sample of more than 11,000 adults and children, and data from five cycles of NHANES (National Health and Nutrition Examination Survey) study from 2001–2010 were used. MEP, MBzP, and MnBP were detected in 98% of participants, while MiBP was detected in 72% of participants in the period 2001–2002 and in 96% participants in the period 2009–2010.

Earlier, Stahlhut et al.²¹ concluded that the urine of more than 95% of participants was positive with the following phthalate metabolites: MEP, MBP, and MBzP, while 80% of participants had MEHP in urine. In the same study, MEP metabolite had noticeably the highest level, followed by MBP and MBzP, while MEHP had the lowest level. In this research, MEHP (detected in 23.7% of cases) and MEP (in 22.4%) were the most represented in the urine sample. The high frequency of the detection of MEHP could be due to the wide use of products that contain di-(2-ethylhexyl)phthalate (DEHP), such as plastic food packaging, toys, and many other household products, while MEP presence is probably the consequence of the increased use of different cosmetic and beauty products, as well as medications, containing diethyl phthalate (DEP)²².

Similar to the findings of previous studies, Hoppin et al.²³ found the highest levels of MEP, MBzP, MBP, and MEHP in the two consecutive morning urine samples of 46 Afro-American women. There was no significant difference in the level of phthalate metabolites between the two urine samples, which indicates that urine is a suitable medium for the measurement of phthalates with a short half-life. Additionally, high urinary levels of MEP, MnBP, and MBzP were found in a study conducted on 289 adult persons by

Blount et al.²⁴, while MEHP was measured in much lower concentrations.

Similar to this research, detectable levels of MEP, MBP, MBzP, MEHP, MiNP, MOP, and MCHP were found in the urine sample of 2,540 volunteers, but with a higher frequency of detection (75%)²⁵. A possible reason for the difference in the distribution is the much smaller sample size in our research (308 vs 2,540 volunteers).

When exposure to six urinary phthalate metabolites was examined in 370 healthy Czech preschool and school children, MEHP, mono (2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono (2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), MBzP, MiBP, and mono-n-butyl phthalate (MnBP) were analyzed. Among all samples, the two latter mono-butyl phthalate isoforms dominated²⁶.

Comparable to similar studies were the results obtained by Frederiksen et al.²⁷, who conducted research on 60 young men to examine the correlation between 13 phthalates metabolites levels in different mediums, such as urine, semen, and serum. DEHP phthalate metabolites, accompanied by MEP, MiBP, MBzP, and MnBP, were detected in the urine samples in the highest amount.

In this research, MEP and MEHP were the most frequently detected metabolites in the control, obese, and T2DM groups. Significant differences were observed for MEP frequency between healthy and T2DM participants ($p = 0.025$). Regarding MEHP, a borderline significant difference was observed between the control and T2DM group ($p = 0.064$), and between the obese and control group ($p = 0.096$).

It is known that activation of PPAR receptors plays an important role in different steps of glucose homeostasis, including insulin secretion and insulin resistance. It can also affect circulating levels of lipids thereby modulating the quantity of subcutaneous and visceral fat. Phthalate metabolites are well-known ligands to PPAR receptors and, therefore, could influence both homeostases of glucose and lipid metabolism. Through the PPAR-signaling pathways deterioration, phthalate metabolites could contribute to the development of obesity and diabetes²⁸. The precise mechanism by which phthalates influence these PPAR-mediated actions is expected to be explained with further experiments.

Limitations and advantages of the study

This study focused only on a middle-aged cluster of white (Caucasian) persons, hence, the results can not be extrapolated to other ethnic and other age groups. Being conducted as a cross-sectional study, this research has a risk of selection bias. Thereby, further studies are needed to confirm the present data. Further studies are also needed for clustering the geographical, age, ethnic, sexual, and other characteristics.

In the current study, urine was used as a matrix for measurements of phthalate metabolites. The advantage of urinary measurements is that apart from low cost and noninvasive methods of obtaining samples, usually higher

levels are found compared with serum and, thereby, more phthalate metabolites could be measured above the lower detection limit.

Conclusion

Approximately half of the examined participants (50.32%) had at least one phthalate metabolite in their urine sample. Our study showed that the most abundant phthalate metabolite present in the group of obese participants was MEP, while MEHP was the most common phthalate metabolite in the T2DM group. A group of healthy individuals had the highest percentage of presence of MEP amongst examined phthalate metabolites. The obtained

results indicate that in the Vojvodina region, both healthy adults and those with metabolic disorders such as obesity and newly diagnosed T2DM are predominantly exposed to widespread DEHP and DEP phthalates.

Further research that will provide more detailed insight into phthalate interference with glucose and lipid metabolism and their influence on the endocrinological balance is needed.

Acknowledgement

This research has been financially supported by the Provincial Secretariat for Science and Technological Development, AP of Vojvodina, Republic of Serbia, Grant No 114-451-2216/2016.

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Received on February 20, 2020

Revised on May 21, 2020

Accepted on September 25, 2020

Online First September, 2020