ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΤΜΗΜΑ ΧΗΜΕΙΑΣ

ΕΡΓΑΣΤΗΡΙΟ ΠΕΡΙΒΑΛΛΟΝΤΙΚΩΝ ΧΗΜΙΚΩΝ ΔΙΕΡΓΑΣΙΩΝ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΜΕΛΕΤΗ ΤΩΝ ΕΠΙΠΕΔΩΝ ΕΚΘΕΣΗΣ ΣΕ ΕΝΔΟΚΡΙΝΙΚΟΥΣ ΔΙΑΤΑΡΑΚΤΕΣ ΤΗΣ ΠΛΗΘΥΣΜΙΑΚΗΣ ΟΜΑΔΑΣ (ΚΟΟΡΤΗΣ) ΜΗΤΕΡΑΣ-ΠΑΙΔΙΟΥ «ΡΕΑ» (ΚΡΗΤΗ)

ΑΝΤΩΝΗΣ ΜΥΡΙΔΑΚΗΣ

Υπεύθυνος Καθηγητής: Ευριπίδης Γ. Στεφάνου

ΗΡΑΚΛΕΙΟ 2015

UNIVERSITY OF CRETE

DEPARTMENT OF CHEMISTRY

ENVIRONMENTAL CHEMICAL PROCESSES LABORATORY

DOCTORAL THESIS

EVALUATION OF EXPOSURE TO ENDOCRINE DISRUPTORS AMONG MOTHER-CHILD PAIRS IN GREECE (RHEA COHORT)

ANTONIS MYRIDAKIS

Thesis Supervisor: Euripides G. Stephanou

HERAKLION 2015

Εξεταστική Επιτροπή

Ευριπίδης Γ. Στεφάνου

Καθηγητής Τμήματος Χημείας (Επιβλέπων)

Γεώργιος Βασιλικογιαννάκης

Καθηγητής Τμήματος Χημείας

Κυριακή Θερμού

Καθηγήτρια Σχολής ΕπιστημώνΥγείας

Μιχαήλ Ορφανόπουλος

Καθηγητής Τμήματος Χημείας

Σπυρίδων Περγαντής

Καθηγητής Τμήματος Χημείας

Απόστολος Σπύρος

Επίκουρος Καθηγητής Τμήματος Χημείας

Νικόλαος Χανιωτάκης

Καθηγητής Τμήματος Χημείας

Ευχαριστίες

Ενώ πλησιάζει το κλείσιμο ενός κύκλου έξι ετών στο Εργαστήριο Περιβαλλοντικών Χημικών Διεργασιών, όπου εντάχθηκα σαν προπτυχιακός φοιτητής στα πλαίσια της διπλωματικής μου εργασίας και φεύγω με την ολοκλήρωση της διδακτορικής μου διατριβής, θα ήθελα να ευχαριστήσω μέσα από τις παρακάτω γραμμές μια σειρά από ανθρώπους.

Αναντίρρητα, το μεγαλύτερο ευχαριστώ ανήκει τον επιβλέποντα της διδακτορικής μου διατριβής, καθηγητή του τμήματος Χημείας και πρύτανη του Πανεπιστημίου Κρήτης, Ευριπίδη Γ. Στεφάνου. Θεωρώ σημαντικότερο από όλα το ότι σε αυτόν χρωστάω την αγάπη μου για την έρευνα. Στάθηκε ένας εξαιρετικός δάσκαλος και παράλληλα η αλληλεπίδραση μαζί του ήταν πάντα ιδιαίτερα ευχάριστη και σε ανθρώπινο επίπεδο.

Στη συνέχεια, ευχαριστώ τα μέλη της εξεταστικής επιτροπής που αξιολόγησαν το ερευνητικό μου έργο, τον καθ. Γεώργιο Βασιλικογιαννάκη, την καθ. Κυριακή Θερμού (Ιατρική Π.Κ.), τον καθ. Μιχαήλ Ορφανόπουλο, τον καθ. Σπύρο Περγαντή, τον επ. καθ. Απόστολο Σπύρο και τον καθ. κ. Νικόλαο Χανιωτάκη και συνολικά το τμήμα Χημείας για την υλικοτεχνική υποδομή και τους ανθρώπους του. Στον κύριο Περγαντή οφείλω ένα πιο ιδιαίτερο ευχαριστώ γιατί πέρα από τη συμμετοχή του στις εξεταστικές επιτροπές μου, έμαθα πολλά όπως και βοηθήθηκα σε θέματα φασματομετρίας μάζας, πάντα με προθυμία.

Για την πολύ ευχάριστη καθημερινότητα στο Εργαστήριο Περιβαλλοντικών Χημικών Διεργασιών πρέπει να αναφερθώ σε πολλούς ανθρώπους αλλά ενδεικτικά, ευχαριστώ πολύ το Δρ. Αντώνη Κουβαράκη για τη συνεργασία και τη βοήθεια του σε όλη τη διάρκεια των μεταπτυχιακών μου χρόνων όπως και την εξαιρετική σχέση που έχουμε, τη Δρ. Μαρία Αποστολάκη που με καθοδήγησε στα πρώτα βήματα μου στο εργαστήριο, τη Δρ. Σοφία Καργάκη και τον υποψήφιο διδάκτορα Μηνά Ιακωβίδη που τους θεωρώ καλούς μου φίλους πλέον και όλα τα παιδιά με τα οποία συνεργάστηκα αυτά τα χρόνια.

Η πραγματοποίηση της παρούσας διατριβή βασίστηκε σε βαθμό στη συνεργασία με τη μελέτη «ΡΕΑ» του τομέα Κοινωνικής Ιατρικής της Ιατρικής σχολής του Π.Κ.. Θα ήθελα να ευχαριστήσω τους υπευθύνους της μελέτης, καθηγητή Μανόλη Κογεβίνα (CREAL. Βαρκελώνη) και την επ. καθ. Λήδα Χατζή, καθώς και όλους τους συνεργάτες και συμμετέχοντες στη μελέτη.

Τέλος, ευχαριστώ τη μητέρα μου και το θείο μου για την αγάπη τους και τη στήριξη στις επιλογές μου, τους φίλους μου και τη Νένα που βαδίσαμε χέρι-χέρι την πορεία μας μέχρι τώρα στο πανεπιστήμιο.

ΒΙΟΓΡΑΦΙΚΟ ΣΗΜΕΙΩΜΑ

ΕΚΠΑΙΔΕΥΣΗ

2004-2009: Πτυχίο Χημείας, Σχολή Θετικών και Τεχνολογικών Επιστημών, Πανεπιστήμιο Κρήτης.
Βαθμός: **7.21/10 (Λίαν καλώς)**.

Διπλωματική εργασία: "*Ποιοτικός και ποσοτικός προσδιορισμός μονο- και δι- καρβοξυλικών οξέων σε περιοχή υποβάθρου της Ανατολικής Μεσογείου (Φινοκαλιά)*". Βαθμός: **10/10 (Άριστα)**.

Επιβλέπων: Καθηγητής Ευριπίδης Γ. Στεφάνου

2009-2011: Μεταπτυχιακό Δίπλωμα Ειδίκευσης στις "**Επιστήμες και Μηχανική Περιβάλλοντος**", Τμήμα Χημείας, Σχολή Θετικών και Τεχνολογικών Επιστημών, Πανεπιστήμιο Κρήτης.

Μαθήματα (Βαθμός):

Περιβαλλοντική Αναλυτική Χημεία Ι: Ανάλυση Οργανικών Ρυπαντών (9/10)

Περιβαλλοντική Αναλυτική Χημεία ΙΙ: Ιόντα και Στοιχεία (8.5/10)

Φασματοσκοπία NMR: Θεωρία και Εφαρμογές (9/10)

Φασματοσκοπία IR και RAMAN (9/10)

Φυσική και Χημεία της Ατμόσφαιρας: Κλιματικές Αλλαγές (9.5/10)

Επεξεργασία Υγρών Αποβλήτων (8.5/10)

Ειδικό Σεμινάριο (υποχρεωτικό): Νομικά Περιβάλλοντος

Τίτλος μεταπτυχιακής διατριβής (υποχρεωτική): *«Ανάπτυξη μεθόδου προσδιορισμού των μεταβολιτών των φθαλικών εστέρων στα ούρα με χρήση υγρής χρωματογραφίας-δίδυμης φασματομετρίας μάζας με ιονισμό ηλεκτροψεκασμού»*. Βαθμός διατριβής: **Άριστα.** Επιβλέπων: Καθηγητής Ευριπίδης Γ. Στεφάνου

Σεπτέμβριος 2011 – Απρίλιος 2015: Διδακτορική διατριβή. Εργαστήριο Περιβαλλοντικών Χημικών Διεργασιών (Ε.ΠΕ.ΧΗ.ΔΙ.), Τμήμα Χημείας, Σχολή Θετικών και Τεχνολογικών Επιστημών, Πανεπιστήμιο Κρήτης. Επιβλέπων: Καθηγητής Ευριπίδης Γ. Στεφάνου

Τίτλος διδακτορικής διατριβής: *«Μελέτη των επιπέδων έκθεσης σε ενδοκρινικούς διαταράκτες της πληθυσμιακής ομάδας (κοόρτης) μητέρας-παιδιού ΡΕΑ (Κρήτη)»*.

ΕΡΕΥΝΗΤΙΚΗ ΕΜΠΕΙΡΙΑ

11/2009-02/2013: Συμμετοχή ως ερευνητής στο Ευρωπαϊκό ερευνητικό πρόγραμμα "Envirogenomarkers - Genomic Biomarkers of Environmental Health" (FP7-ENV-2008-1, Grant Agreement No.226756). Αρμοδιότητες: Ανάπτυξη μεθόδων προσδιορισμού μεταβολιτών φθαλικών εστέρων και παραβενίων σε ανθρώπινα ούρα (ενζυμική υδρόλυση συζευγμένων μεταβολιτών, SPE και ανίχνευση με HPLC-ESI-MS/MS (MRM)). Εφαρμογή σε 239 ζευγάρια μητέρας-παιδιού (εγκυμοσύνη και 2.5 έτη). Στατιστική επεξεργασία (εκτίμηση έκθεσης). Συνεργασία με την Επιδημιολογική ομάδα της Ιατρικής σχολής (Πανεπιστήμιο Κρήτης, Ελλάδα) για τη συσχέτιση της περιβαλλοντικής έκθεσης με κλινικά δεδομένα.

15/10/2013-02/2015: Συμμετοχή ως ερευνητής στο ερευνητικό "RHEA Plus" (ΑΡΙΣΤΕΙΑ, ΕΣΠΑ 2007-2013, Α.Ο. 380193). Αρμοδιότητες: Ανάπτυξη μεθόδων προσδιορισμού μεταβολιτών οργανοφωσφορικών φυτοφαρμάκων και δισφαινόλης-Α σε ανθρώπινα ούρα (ενζυμική υδρόλυση συζευγμένων μεταβολιτών, SPE και ανίχνευση με HPLC-ESI-MS/MS (MRM)). Εκτίμηση επιπέδων έκθεσης 500 παιδιών 4 ετών σε μεταβολίτες

φθαλικών εστέρων, οργανοφωσφορικών φυτοφαρμάκων, παραβενίων και δισφαινόλης-Α. Στατιστική επεξεργασία (εκτίμηση έκθεσης). Συνεργασία με την Επιδημιολογική ομάδα της Ιατρικής σχολής (Πανεπιστήμιο Κρήτης, Ελλάδα) για τη συσχέτιση της περιβαλλοντικής έκθεσης με κλινικά δεδομένα.

ΕΡΕΥΝΗΤΙΚΑ ΕΝΔΙΑΦΕΡΟΝΤΑ

- Βιοαναλυτική χημεία
- Μοριακή φασματομετρία μάζας συζευγμένη με τεχνικές διαχωρισμού
- Μέτρηση ιχνοποσοτήτων (trace analysis)
- Μελέτη βιοδεικτών (biomarkers)
- Προσδιορισμός μεταβολιτών
- Εκτίμηση έκθεσης

ΑΝΑΛΥΤΙΚΕΣ ΤΕΧΝΙΚΕΣ

LC-MS/MS: ESI/APCI. Triple quadrupole/iontrap. MRM/SIM/Full scan/Neutral loss scan/Product ion scan

GC-MS: Electron ionization.

Διάφορες τεχνικές απομόνωσης και καθαρισμού οργανικών ενώσεων σε βιολογικά υγρά και περιβαλλοντικά δείγματα: (reversed phase / ion exchange / molecularly imprinted polymer SPE, liquid-liquid extraction, microwave extraction, διάφορες τεχνικές παραγοντοποίησης κλπ.)

ΕΠΙΣΤΗΜΟΝΙΚΕΣ ΔΗΜΟΣΙΕΥΣΕΙΣ ΣΕ ΔΙΕΘΝΗ ΠΕΡΙΟΔΙΚΑ ΜΕ ΚΡΙΤΕΣ

1. Karageorgou, E.; Myridakis, A.; Stephanou, E. G.; Samanidou, V., Multiresidue LC-MS/MS analysis of cephalosporins and quinolones in milk following ultrasound-assisted matrix solid-phase dispersive extraction combined with the quick, easy, cheap, effective, rugged, and safe methodology. *J Sep Sci* **2013,** *36* (12), 2020-7.

2. Riga, M.; Tsakireli, D.; Ilias, A.; Morou, E.; Myridakis, A.; Stephanou, E. G.; Nauen, R.; Dermauw, W.; Van Leeuwen, T.; Paine, M.; Vontas, J., Abamectin is metabolized by CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in Tetranychus urticae. *Insect Biochem Mol Biol* **2014,** *46*, 43-53.

3. Vardavas, C. I.; Karabela, M.; Agaku, I. T.; Matsunaga, Y.; Myridakis, A.; Kouvarakis, A.; Stephanou, E. G.; Lymperi, M.; Behrakis, P. K., Secondhand smoke exposure within semi-open air cafes and tobacco specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) concentrations among nonsmoking employees. *Int J Occup Med Environ Health* **2014,** *27* (5), 875-81.

4. Nauen, R.; Wölfel, K.; Lueke, B.; **Myridakis, A.;** Tsakireli D.; Roditakis, E.; Tsagkarakou, A.; Stephanou, E.G.; Vontas, J., Development of a lateral flow test to detect metabolic resistance in Bemisia tabaci mediated by CYP6CM1, a cytochrome P450 with broad spectrum catalytic efficiency. *Pestic Biochem Phys,* **2015** *(in press)*

5. **Myridakis, A.;** Balaska, E.; Gkaitatzi, C.; Kouvarakis, A.; Stephanou, E. G., Determination and separation of bisphenol A, phthalate metabolites and structural isomers of parabens in human urine with conventional

high-pressure liquid chromatography combined with electrospray ionisation tandem mass spectrometry. *Anal Bioanal Chem*, **2015,** DOI 10.1007/s00216-015-8497-5.

6. **Myridakis, A.;** Fthenou, E.; Balaska, E.; Kogevinas, M.; Stephanou, E.G. Phthalate esters and parabens exposure among mothers and their children in Greece (RHEA cohort). *(submitted to Environment International on 11-Jan-2015)*

7. **Myridakis, A**.; Apostolaki, M.; Katsikantami I.; Perraki, A.; Chalkiadaki G., Kogevinas M.; Chatzi L.; Stephanou, E.G. Occurrence of bisphenol-A, parabens and phthalate metabolites in urine of preschool-age children in Greece (RHEA cohort) and of phthalates in drinking water and indoor air of the study area. *(Submitted to Chemosphere on 20-Feb-2015)*

8. **Myridakis, A.;** Fotou, M.; Chalkiadaki G.; Kogevinas M.; Chatzi L.; Stephanou, E.G. Concentration levels of dialkyl phosphate metabolites of organophosphorus pesticides among preschool-age children in Greece (RHEA cohort) *(to be submitted to Chemosphere)*

<u>ΣΥΝΕΔΡΙΑ</u>

1. Envirogenomarkers Annual Symposium: **Myridakis, A.;** Kouvarakis, A.; Stephanou, E.G. Quantitative detection of urinary phthalate metabolites in humans using liquid chromatography-electrospay ionization tandem mass spectrometry. Oral Presentation. Santorini, Greece, 03-04 March, **2010.**

2. Member of the organizing committee of 16th Hellenic and Cypriot Chemistry Post Grads Conference. Protaras, Cyprus, 23-27 June 2010

3. President of the organizing committee of 17th Hellenic Chemistry Post Grads Conference. Heraklion, Greece, 15-18 July **2011.**

4. Envirogenomarkers Annual Symposium: **Myridakis, A.;** Gaitatzi, C.; Perraki, A.; Kouvarakis, A.; Apostolaki, M.; Stephanou E.G. Exposure levels to phthalate esters: Mother-Child "Rhea" (Crete) Cohort and some related studies. Oral Presentation. Turin, Italy, 19 March, **2012.**

5. Karageorgou, E**.; Myridakis, A.;** Stephanou, E.G.; Samanidou, V. Multi-residue LC-MS/MS analysis of cephalosporins and quinolones in milk following ultrasound assisted matrix solid phase dispersive extraction combined with QuEChERS methodology. 8th International Conference "IMA 2013-Instrumental Methods of Analysis-Modern Trends and Applications". Poster. Thessaloniki, Greece, 15-19 September **2013.**

6. Vardavas C, Karampela M, Agaku I, **Myridakis A,** Kouvarakis A, Stephanou E, et al. 2013. "Occupational SHS Exposure within Semi-Open Air Venues and Tobacco Specific Carcinogen Uptake". Chest 2013. Poster. Chicago, Illinois, U.S.A. 26–31 October, **2013.**

<u>ΔΙΔΑΚΤΙΚΗ ΕΜΠΕΙΡΙΑ</u>

2009-2010 (Χειμερινό εξάμηνο): Βοηθός ασκήσεων προπτυχιακού μαθήματος Χημεία Περιβάλλοντος I: Υδατική Χημεία

2010-2011 (Χειμερινό εξάμηνο): Προετοιμασία σεμιναρίου "Εφαρμογής υγρής χρωματογραφίας συζευγμένης με δίδυμη φασματομετρία μαζών στην ανάλυση μεταβολιτών φθαλικών εστέρων στα ανθρώπινα ούρα (Μεταπτυχιακό μάθημα: Περιβαλλοντική Αναλυτική Χημεία I: Ανάλυση Οργανικών Ρυπαντών)"

2011-2012 (Χειμερινό εξάμηνο): Βοηθός ασκήσεων προπτυχιακού μαθήματος Αναλυτική Χημεία Ι

2013-2014 (Εαρινό εξάμηνο): Βοηθός ασκήσεων προπτυχιακού μαθήματος Χημεία Περιβάλλοντος Ι: Υδατική Χημεία

Η/Υ

Λειτουργικά συστήματα: Windows, MacOS/iOS, Android

Διάφορα λογισμικά: MS Office, ChemBioDraw, SPSS, Statistica, XLStat, Origin

Στατιστικές τεχνικές: PCA, περιγραφική στατιστική, παραμετρικά/μη παραμετρικά tests, Spearman-Pearson συσχετίσεις κλπ.

ΓΛΩΣΣΕΣ

Ελληνικά: Μητρική

Αγγλικά: First Certificate in English, University of Cambridge

Γερμανικά: Zertifikat Deutsch, Goethe Institut

EDUCATION

2004-2009: B.Sc. in Chemistry, Faculty of Sciences and Engineering, University of Crete.
Grade: **7.21/10 (Very Good)**.
Diploma Thesis: "*Qualitative and quantitative detection of monocarboxylic and dicarboxylic acids in aerosol samples from a background area of Eastern Mediterranean (Finokalia)*".   Grade: **10/10** (**Excellent**)**.**
Supervisor: Professor Dr. Euripides G. Stephanou
2009-2011:   Master of Science Degree in "**Environmental Science and Engineering**", Chemistry Department, Faculty of Sciences and Engineering, University of Crete.
Courses (Grade):
Environmental Analytical Chemistry I: Analysis of Organic Pollutants (9/10)
Environmental Analytical Chemistry II: Ions and Elements (8.5/10)
NMR Spectroscopy: Theory and Applications (9/10)
IR and RAMAN Spectroscopy (9/10)
Atmospheric Chemistry and Physics: Climatic Changes (9.5/10)
Liquid Waste Processing (8.5/10)
Special Seminar (compulsory): Environmental Legislation
M.Sc. Thesis title (compulsory):   "*Detection of urinary phthalate metabolites in humans using liquid chromatography – electrospray ionization tandem mass spectrometry*".   Grade: **Excellent**.   Supervisor: Professor Dr. Euripides G. Stephanou
September 2011 – April 2015 (expected): Phd Thesis: Environmental Chemical Processes Laboratory, Chemistry Department, Faculty of Sciences and Engineering, University of Crete. Supervisor: Professor Dr. Euripides G. Stephanou.
PhD Thesis title: "*Evaluation of Endocrine Disruptor Exposure among Mother-Child pairs in Greece (RHEA cohort)*"

RESEARCH EXPERIENCE

11/2009-02/2013: Participation as researcher in EU funded research program "Envirogenomarkers - Genomic Biomarkers of Environmental Health" (FP7-ENV-2008-1, Grant Agreement No.226756). Task: Determination of urinary parabens and phthalate metabolite method development (metabolites hydrolysis, clean up with SPE and HPLC-ESI-MS/MS detection), application to 239 mother-child pairs (samples during pregnancy and at two year old children). Statistical analysis of the results (exposure assessment). Collaboration with the Epidemiology group of Medical School (University of Crete, Greece) for the integration of environmental exposure and clinical examinations results.

15/10/2013-02/2015: Participation as researcher in research program "RHEA Plus" in the frame of National Strategic Reference Framework (NSRF) 2007–2013, funded by EU and Greek government (Grant Agreement No. 380193). Task: Determination of urinary alkyl phosphates and bisphenol-A method development (metabolites hydrolysis, clean up with SPE and HPLC-ESI-MS/MS detection). Determination of phthalate

metabolites, parabens, bisphenol-A and alkyl phosphates in 4-year old children (five hundred subjects). Statistical analysis of the results (exposure assessment). Collaboration with the Epidemiology group of Medical School (University of Crete, Greece) for the integration of environmental exposure and clinical examinations results.

RESEARCH INTERESTS

- Bioanalytical chemistry
- Molecular mass spectrometry hyphenated with chromatography
- Metabolite determination
- Biomarker studies
- Trace analysis
- Exposure assessment

ANALYTICAL TECHNIQUES

LC-MS/MS: Liquid chromatography-mass spectrometry and –tandem mass spectrometry operated with electrospray and atmospheric pressure chemical ionisation

GC-MS: Gas chromatography-mass spectrometry, operated with electron ionisation

Various sample preparation and isolation techniques for organic compounds in environmental samples and human fluids: (reversed phase / ion exchange / molecularly imprinted polymer solid phase extraction, liquid-liquid extraction, microwave extraction, various derivatisation techniques etc.)

PEER REVIEWED JOURNAL ARTICLES

1. Karageorgou, E.; Myridakis, A.; Stephanou, E. G.; Samanidou, V., Multiresidue LC-MS/MS analysis of cephalosporins and quinolones in milk following ultrasound-assisted matrix solid-phase dispersive extraction combined with the quick, easy, cheap, effective, rugged, and safe methodology. *J Sep Sci* **2013,** *36* (12), 2020-7.
2. Riga, M.; Tsakireli, D.; Ilias, A.; Morou, E.; Myridakis, A.; Stephanou, E. G.; Nauen, R.; Dermauw, W.; Van Leeuwen, T.; Paine, M.; Vontas, J., Abamectin is metabolized by CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in Tetranychus urticae. *Insect Biochem Mol Biol* **2014,** *46*, 43-53.
3. Vardavas, C. I.; Karabela, M.; Agaku, I. T.; Matsunaga, Y.; Myridakis, A.; Kouvarakis, A.; Stephanou, E. G.; Lymperi, M.; Behrakis, P. K., Secondhand smoke exposure within semi-open air cafes and tobacco specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) concentrations among nonsmoking employees. *Int J Occup Med Environ Health* **2014,** *27* (5), 875-81.
4. Nauen, R.; Wölfel, K.; Lueke, B.; **Myridakis, A.;** Tsakireli D.; Roditakis, E.; Tsagkarakou, A.; Stephanou, E.G.; Vontas, J., Development of a lateral flow test to detect metabolic resistance in Bemisia tabaci mediated by CYP6CM1, a cytochrome P450 with broad spectrum catalytic efficiency. *Pestic Biochem Phys,* **2015** *(in press)*

5. **Myridakis, A.;** Balaska, E.; Gkaitatzi, C.; Kouvarakis, A.; Stephanou, E. G., Determination and separation of bisphenol A, phthalate metabolites and structural isomers of parabens in human urine with conventional high-pressure liquid chromatography combined with electrospray ionisation tandem mass spectrometry. *Anal Bioanal Chem*, **2015** (DOI 10.1007/s00216-015-8497-5)

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2. Member of the organizing committee of 16th Hellenic and Cypriot Chemistry Post Grads Conference. Protaras, Cyprus, 23-27 June **2010.**

3. President of the organizing committee of 17th Hellenic Chemistry Post Grads Conference. Heraklion, Greece, 15-18 July **2011.**

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TEACHING EXPERIENCE

2009-2010 (Winter semester): Teaching assistant at the courses of Environmental Chemistry I: Aquatic Chemistry

2010-2011 (Winter semester): Preparation of seminar "Application of LC-MS/MS in analysis of phthalate metabolites in human urine (Postgraduate Course: Environmental Analytical Chemistry I: Analysis of Organic Pollutants)"

2011-2012 (Winter semester): Teaching assistant at the courses of Analytical Chemistry I

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ΠΕΡΙΛΗΨΗ

Οι φθαλικοί εστέρες (phthalate esters, PEs), η δισφαινόλη-Α (bisphenol-A, BPA) και τα παραβένια (parabens, PBs) χρησιμοποιούνται ευρέως σε πάρα πολλά προϊόντα καθημερινής χρήσης. Παρουσιάζουν ενδοκρινική δραστικότητα και συνδέονται με αρνητικές επιδράσεις στη υγεία. Ο ανθρώπινος οργανισμός, αφού έρθει σε επαφή με αυτές τις ενώσεις, τις μεταβολίζει και τις αποβάλει κυρίως μέσω των ούρων. Τα επίπεδα των μεταβολιτών στα ούρα αποτελούν βιοδείκτες έκθεσης.

Αναπτύχθηκε μια μέθοδος προσδιορισμού οκτώ μεταβολιτών των PE, έξι PB και της BPA με χρήση υγρής χρωματογραφίας συζευγμένης με διαδοχική φασματομετρία μαζών (liquid chromatography-tandem mass spectrometry, LC-MS/MS). Για πρώτη φορά επετεύχθη: Ι) ένα κοινό πρωτόκολλο επεξεργασίας ανθρώπινων ούρων για τον προσδιορισμό μεταβολιτών των PEs, PBs και BPA, ΙΙ) ο διαχωρισμός των ισο- / κ- μορφών του πρόπυλο- και βούτυλο- παραβενίου με χρήση συμβατικής χρωματογραφικής στήλης υψηλής απόδοσης.

Η μέθοδος εφαρμόστηκε στην ανάλυση δειγμάτων από διακόσιες τριάντα εννέα (239) εγκυμονούσες γυναίκες, τα ισάριθμα (239) παιδιά τους στην ηλικία των 2.5 ετών και από πεντακόσια (500) 4-χρονα παιδιά. Όλα τα άτομα της μελέτης είναι μέλη της κοόρτης «ΡΕΑ». Τα επίπεδα συγκεντρώσεων των μεταβολιτών των PEs και της BPA ήταν συγκρίσιμα με αντίστοιχες μελέτες, που διεξάχθηκαν σε χώρες της Ευρώπης και των Η.Π.Α., ενώ τα επίπεδα των PBs βρέθηκαν υψηλότερα σε σχέση με μελέτη από τη Δανία. Η ημερήσια πρόσληψη (Daily Intake, DI) κατά την εγκυμοσύνη ήταν υψηλότερη σε σχέση με τα παιδιά για όλες τις ενώσεις που εξετάστηκαν, εκτός από το δι-αίθυλο-έξυλο-εστέρα του φθαλικού οξέος (di-2-ethylhexyl phthalate, DEHP) και την BPA. Τα επίπεδα της παραμέτρου DI ήταν χαμηλότερα στα παιδιά 4-ετών σε σχέση με αυτά των 2.5 ετών. Ασθενείς συσχετίσεις (two-tailed spearman; CC 0.1-0.2; p<0.01) παρατηρήθηκαν στις συγκεντρώσεις εγκυμοσύνης/παιδιών 2.5-ετών των δι-αίθυλο-φθαλικού εστέρα (di-ethyl phthalate, DEP), δι-βούτυλο-φθαλικού εστέρα (di-n-butyl phthalate, DnBP), βούτυλο-βένζυλο-φθαλικού εστέρα (butyl-benzyl phthalate, BBP) και αίθυλο-παραβενίου (ethyl paraben, EPB). Η έκθεση ομαδοποιήθηκε σε δυο κύριες πηγές: προερχόμενη από πλαστικά υλικά για τους PEs/BPA και από είδη προσωπικής υγιεινής-φροντίδας για τα PBs/DEP(di-ethyl phthalate). Η μελέτη του μεταβολισμού του DEHP έδειξε ότι η έκταση του σχετίζεται με την ηλικία και το φύλο. Η συνεισφορά στην έκθεση στα PEs στην αντίστοιχη ημερήσια πρόσληψη, μέσω του αέρα εσωτερικού χώρου και του πόσιμου νερού, ήταν σε χαμηλά ποσοστά ως προς τη συνολική.

Λέξεις κλειδιά: ενδοκρινικοί διαταράκτες, βιοδείκτες έκθεσης, δισφαινόλη-Α, παραβένια, φθαλικοί εστέρες, κοόρτη μητέρας-παιδιού «ΡΕΑ», υγρή χρωματογραφία, δίδυμη φασματομετρία μάζας, ηλεκτροψεκασμός

ABSTRACT

Phthalate esters (PEs), bisphenol-A (BPA) and parabens (PBs) are used widely in everyday products. They have endocrine disrupting properties and are linked with adverse health effects. Humans exposed to these chemicals, metabolize and excrete them mostly via urine. Urinary metabolite concentrations are used as biomarkers of exposure.

A liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed in order to determine eight PEs metabolites, six PBs and BPA in human urine. For the first time: I) all three categories of the above endocrine disruptors were simultaneously extracted from human urine and II) the separation of two pairs of structural isomers, namely, iso-/n- butyl paraben and propyl paraben was achieved with a conventional reversed phase LC column.

The developed method was applied to samples from two hundred and thirty nine (239) pregnant women, their 2.5-year old children (239), and five hundred (500) 4-year old children, all subjects of the "Rhea" cohort (Crete). Concentration levels of BPA and PEs metabolites were comparable with other studies worldwide. PBs levels were higher compared to a relevant study conducted in Denmark. We observed for all examined compounds, except for di-2-ethylhexyl phthalate (DEHP) and BPA, higher median daily intake (DI_u) for mothers than for their children. DI_u for 4-year children was lowest compared to the corresponding for 2.5-year old children. Low correlations (two-tailed spearman; CC 0.1-0.2; $p<0.01$) were observed for di-ethyl phthalate (DEP), di-n-butyl phthalate (DnBP), butyl-benzyl phthalate (BBP) and ethyl paraben (EPB) concentrations between mothers and their children. Exposure was assigned to two main sources: a) plastics related to PE-BPA and, b) to personal care-hygiene products related to PB and DEP (di-ethyl phthalate). DEHP metabolism seems to be related to age and sex. Indoor air and drinking water PEs daily intake represented a small fraction of the total daily intake.

Keywords: endocrine disruptors; exposure biomarkers; bisphenol-A; parabens; phthalate esters; mother-child cohort Rhea; preschool-age children; liquid chromatography, electrospray ionisation, tandem mass spectrometry.

ΠΕΡΙΕΧΟΜΕΝΑ

CONTENTS

Συντομογραφίες

APCI: Χημικός Ιονισμός υπό Ατμοσφαιρική Πίεση, Atmospheric Pressure Chemical Ionization

BBP: 1-βούτυλο-2-βένζυλο-εστέρας του φθαλικού οξέος, butyl-benzyl phthalate

BPA: δισφαινόλη-Α, bisphenol-A

DEHP: δι-αίθυλο-έξυλο-εστέρας του φθαλικού οξέως, di-2-ethylhexyl phthalate

DEP: δι-αίθυλο εστέρας του φθαλικού οξέος, di-ethyl phthalate

DI: Ημερήσια Πρόσληψη, Daily Intake

DiBP: δι-ίσο-βούτυλο-εστέρας του φθαλικού οξέος, di-iso-butyl phthalate

DnBP: δι-κ-βούτυλο-εστέρας του φθαλικού οξέος, di-n-butyl phthalate

EDs: Ενδοκρινικοί Διαταράκτες, Endocrine Disruptors

EFSA: Ευρωπαϊκή Αρχή Ασφάλειας Τροφίμων, European Food Safety Authority

EPB: αίθυλο-παραβένιο, ethyl paraben

ESI: Ηλεκτροψεκασμός, Electrospray ionization

HPLC-MS/MS: High Performance Liquid Chromatography Tandem Mass Spectrometry, Υγρή Χρωματογραφία Υψηλής Απόδοσης συζευγμένη με Διαδοχική Φασματομετρία Μαζών

isoBPB: ίσο-βούτυλο-παραβένιο, iso-butyl paraben

isoPPB: ίσο-πρόπυλο-παραβένιο, iso-propyl paraben

mBzP: μόνο-βένζυλο-εστέρας του φθαλικού οξέος, mono-benzyl phthalate

mEHHP: μόνο-2-αίθυλο-5-όξο-έξυλο-εστέρας του φθαλικού οξέος, mono-2-ethyl-5-hydroxy-hexyl phthalate

mEHP: μόνο-2-αίθυλο-έξυλο-εστέρας του φθαλικού οξέος, mono-2-ethyl-hexyl phthalate

mEOHP: μόνο-2-αίθυλο-5-ύδροξυ-έξυλο-εστέρας του φθαλικού οξέος, mono-2-ethyl-5-oxo-hexyl phthalate

mEP: μόνο-2-αίθυλο -εστέρας του φθαλικού οξέος, mono-ethyl phthalate

mLOD: Όρια Ανίχνευσης Μεθόδου, Method Limits of Detection

mnBP: μόνο-2-κ-βούτυλο-εστέρας του φθαλικού οξέος, mono-n-butyl phthalate

MPB: μέθυλο-παραβένιο, methyl paraben

nBPB: κ-βούτυλο-παραβένιο, n-butyl-paraben

nPPB: κ-πρόπυλο-παραβένιο, n-propyl paraben

PBs: Παραβένια, Parabens

PCA: Ανάλυση Κυρίων Συνιστωσών, Principal Component Analysis

PEs: Φθαλικοί εστέρες, phthalate esters

RMR: Σχετικός Μεταβολικός Ρυθμός, Relative Metabolic Rate

SPE: Εκχύλιση Στερεάς Φάσης, Solid Phase Extraction

UPLC: Υγρή Χρωματογραφία Υπερύψηλης Απόδοσης, Ultra Performance Liquid Chromatography

USEPA: Αμερικάνικη Υπηρεσία Προστασίας Περιβάλλοντος, U.S. Environmental Protection Agency

1. Εισαγωγή

1.1. Ενδοκρινικοί διαταράκτες

Οι ενδοκρινικοί διαταράκτες (endocrine disruptors, EDs) είναι ουσίες που παρεμβαίνουν στην ομαλή λειτουργία του ορμονικού συστήματος στα θηλαστικά. Οι EDs, αλληλεπιδρούν με φυσικές ορμόνες, όπως την εστραδιόλη, την οιστρόνη και την οιστρόλη, που είναι υπεύθυνες για την ανάπτυξη, τη συμπεριφορά και τη διατήρηση της ομοιόστασης. Προσδένονται σε ορμονικούς υποδοχείς και είτε τους ενεργοποιούν είτε τους απενεργοποιούν με ποικίλες επιδράσεις. Η έκθεση στους ενδοκρινικούς διαταράκτες κατά τα διάρκεια του «προγραμματισμού» του ενδοκρινικού συστήματος μπορεί να οδηγήσει σε μόνιμη αλλαγή της λειτουργίας του. Αντιθέτως, η έκθεση κατά τη διάρκεια της ενηλικίωσης, μπορεί να αντισταθμιστεί από τους φυσιολογικούς ομοιοστατικούς μηχανισμούς και έτσι μπορεί να μην οδηγήσει τελικά σε αντίστοιχα σημαντικές επιδράσεις (Vandenberg et al. 2009).

Πληθώρα μελετών καταδεικνύει χημικές ουσίες με ενδοκρινική δραστικότητα, που περιέχονται σε τεράστιο αριθμό προϊόντων ευρείας κατανάλωσης, όπως πλαστικό, καλλυντικά, φάρμακα και τρόφιμα (Rousselle et al. 2013). Η ανθρώπινη έκθεση διενεργείται μέσω κατάποσης (τροφή), εισπνοής (ατμοσφαιρικός αέρας) ή δερματικής επαφής (προϊόντα που έρχονται σε επαφή με το δέρμα), οι EDs μεταβολίζονται και αποβάλλονται από τον ανθρώπινο οργανισμό κυρίως μέσω των ούρων. Ο άνθρωπος είναι περισσότερο ευάλωτος στις βλαπτικές επιδράσεις τους κατά την έκθεση κατά την εμβρυϊκή ζωή μέσω του πλακούντα και κατά την πρώιμη παιδική ηλικία, καθώς επεμβαίνουν σε διάφορα στάδια της ανάπτυξης (Flint et al. 2012, Heudorf et al. 2007, Kang et al. 2006, Uzumcu and Zachow 2007).

Η δισφαινόλη-A (bisphenol-A, BPA), τα παραβένια (parabens, PBs) και οι φθαλικοί εστέρες (phthalate esters, PEs) είναι από τους σημαντικότερους EDs. Η παγκόσμια ετήσια παραγωγή της BPA ανέρχεται σε 3 εκατομμύρια τόνους, των PEs πάνω από 8 εκατομμύρια τόνους και είναι από τις μεγαλύτερης παραγωγής χημικές ουσίες ενώ τα PBs χρησιμοποιούνται σε πάνω από 13.200 παρασκευές για σχεδόν κάθε καλλυντικό (Crinnion 2010, Elder 1984, Flint et al. 2012).

Οι PEs είναι βιομηχανικά προϊόντα με πολλές εφαρμογές. Οι υψηλού μοριακού βάρους PEs χρησιμοποιούνται ως πλαστικοποιητές και οι χαμηλού μοριακού βάρους σε προϊόντα προσωπικής φροντίδας και στη φαρμακευτική. Μελέτες σε πειραματόζωα συνέδεσαν την έκθεση σε PEs με επιπτώσεις στο αναπαραγωγικό σύστημα και καρκινογένεση. Ο μεταβολισμός τους περιλαμβάνει την υδρόλυση στους αντίστοιχους μονοεστέρες και ακολουθείται είτε σύζευξη μιας γλουκουρονικής ομάδας είτε υδροξυλίωση - περαιτέρω οξείδωση του μονοεστέρα πριν τη σύζευξη. Το σύνολο των μεταβολιτών αποβάλλεται μέσω των ούρων (Heudorf et al. 2007).

Η BPA χρησιμοποιείται κυρίως ως πλαστικοποιητής και έχει συνδεθεί με σειρά αρνητικών επιπτώσεων στον άνθρωπο, όπως αναπαραγωγικές - αναπτυξιακές δυσλειτουργίες, επιδράσεις στο συκώτι κ.α. (Vandenberg et al. 2007). Αποβάλλεται μέσω των ούρων με τη μορφή των γλουκουρονιδιωμένων-σουλφονιωμένων μεταβολιτών της σε ποσοστό 13-28% , καθώς και ελεύθερη (Kang et al. 2006).

Τα PBs χρησιμοποιούνται ως αντιμικροβιακά πρόσθετα κυρίως στα καλλυντικά (περιέχονται σχεδόν σε όλα) (Elder 1984) και έχουν συνδεθεί με τον καρκίνο του μαστού (Byford et al. 2002). Αποβάλλονται μέσω των ούρων είτε ελεύθερα είτε ως γλυκινικά, γλουκουρονιδικά και θειικά συζεύγματα (Abbas et al. 2010).

Η εκτίμηση της έκθεσης (και κατά συνέπεια η σύνδεση της με δυσμενείς επιδράσεις στην υγεία) για τα παραπάνω χημικά γίνεται με τη μέτρηση των επιπέδων των μεταβολιτών τους στα ούρα (βιοδείκτες έκθεσης, biomarkers) (Rousselle et al. 2013).

1.2. Φθαλικοί εστέρες

1.2.1 Ιδιότητες, χρήσεις και πηγές έκθεσης

Οι φθαλικοί εστέρες (phthalate esters, PEs) είναι διεστέρες εστέρες του 1,2 βενζοδικαρβοξυλικού οξέος. Η γενική τους δομή φαίνεται στην Εικόνα 1. Οι υποκατάστατης R και R' συνήθως είναι είτε κυρίως γραμμικές ή διακλαδισμένες άλκυλο- αλυσίδες είτε φαινυλικές, κυκλοαλκυλικές και άλκοξυ- ομάδες .

Οι φθαλικοί εστέρες χαμηλότερου μοριακού βάρους και με μικρές διακλαδώσεις όπως ο φθαλικός δι-αιθυλεστέρας (di-ethyl phthalate ester) χρησιμοποιούνται εκτεταμένα στα καλλυντικά (Wormuth et al. 2006). Το DEP έχει βρεθεί σχεδόν σε όλα τα προϊόντα προσωπικής φροντίδας για βρέφη, παιδιά και ενήλικους. Επιπλέον, ο φθαλικός δι-κ-βουτυλεστέρας (di-n-butyl phthalate ester, DnBP) και ο ο φθαλικός δι-ισο-βουτυλεστέρας (di-iso-butyl phthalate ester, DiBP) είναι συνήθη πρόσθετα σε καλλυντικά για ενηλίκους όπως τα αρώματα, τα σαμπουάν και τα προϊόντα περιποίησης νυχιών.
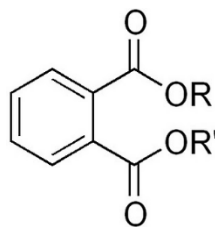
Εικόνα 1. Γενική δομή φθαλικών εστέρων

Οι φθαλικοί εστέρες υψηλότερου μοριακού βάρους όπως ο φθαλικός βουτυλοβενζυλεστέρας (butyl-benzyl phthalate, BBP), ο φθαλικός δι-2-αιθυλοεξυλεστέρας (di-2-ethylhexyl phthalate ester, DEHP) αλλά και τα DiBP και DnBP χρησιμοποιούνται σε πλαστικά, όπως τα δάπεδα από βινύλιο, οι βαφές και άλλα οικοδομικά υλικά, σε παιχνίδια, πλαστικές σακούλες, γάντια, παπούτσια και σε απομιμήσεις δέρματος (Wormuth et al. 2006). Επιπρόσθετα, το DEHP χρησιμοποιείται σαν πλαστικοποιητής σε κάποιες ιατρικές συσκευές όπως οι φιάλες αίματος και οι ενδοφλέβιοι σωλήνες. Χαρακτηριστικά αναφέρεται ότι μερικά μαλακά πλαστικά περιέχουν έως 40% DEHP. Στην Ευρώπη, τα περισσότερα τρόφιμα που έρχονται σε επαφή με πλαστικά περιέχουν DEHP και DBP, ενώ το DEHP περιέχεται σε δημητριακά, ψωμί, κέικ, ξηρούς καρπούς, μπαχαρικά, λάδι σε ποσότητες περίπου μέχρι και τα 10mg/kg. Το 2003, περισσότεροι από 800.000 τόνοι φθαλικών χρησιμοποιήθηκαν στη Δυτική Ευρώπη με το DEHP να καταλαμβάνει το 24%.

Πρακτικά, σχεδόν όλα τα βιομηχανικά καταναλωτικά προϊόντα περιέχουν φθαλικούς εστέρες ή ίχνη τους. Παρόλο που οι συγκεκριμένες ουσίες μεταβολίζονται πολύ γρήγορα, η επιμόλυνση του περιβάλλοντος είναι σημαντική εξαιτίας της εκτεταμένης χρήσης τους και της παρουσίας τους στη σκόνη, στο νερό, στο έδαφος και στον αέρα (Wams 1987, Wormuth et al. 2006).

1.2.2. Μεταβολισμός και επιδράσεις στην υγεία

Οι φθαλικοί διεστέρες μεταβολίζονται ταχύτατα στον οργανισμό του ανθρώπου σε δύο κύριες φάσεις: η πρώτη είναι η υδρόλυση στον αντίστοιχο μονοεστέρα που ακολουθείται από τη δεύτερη φάση, τη σύζευξη με μια γλουκουρονική ομάδα, όπως φαίνεται στην Εικόνα 2 (Calafat et al. 2006).

Πιο αναλυτικά, στη πρώτη φάση ο φθαλικός διεστέρας υδρολύεται στο κύριο μεταβολίτη του, το φθαλικό μονοεστέρα, σε μια διαδικασία που καταλύεται ενζυμικά, από τις λιπάσες και τις εστεράσες στο έντερο και στο παρέγχυμα (ATSDR DEHP 2001, ATSDR DEP 1995, ATSDR DnBP 2001). Θεωρητικά η υδρόλυση θα έπρεπε να μειώνει τα επίπεδα τοξικότητας των φθαλικών αλλά in vivo και in vitro πειράματα έχουν δείξει ότι οι φθαλικοί διεστέρες γίνονται τοξικότεροι όταν υδρολύονται προς τους αντίστοιχους μονοεστέρες (Heindel and Powell 1992).
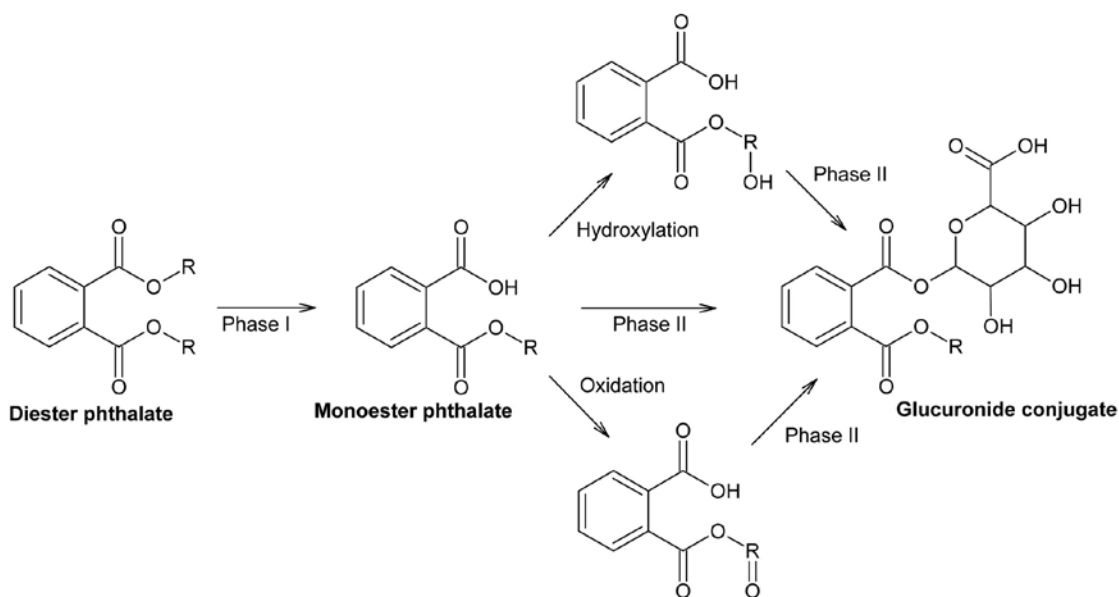
Εικόνα 2. Μεταβολικά μονοπάτια των φθαλικών εστέρων

Οι σχετικά πολικοί και χαμηλού μοριακού βάρους φθαλικοί εστέρες αποβάλονται κυρίως μέσω των ούρων με τη μορφή των μονοεστέρων, ενώ οι φθαλικοί εστέρες υψηλότερου μοριακού βάρους υφίστανται περεταίρω βιομετατροπές, συμπεριλαμβανομένης της υδροξυλίωσης και οξείδωσης τους στη συνέχεια, αποβάλλονται μέσω των ούρων ή των κοπράνων με τη συζευγμένη μορφή τους (Εικόνα 2) (Koch et al. 2004, Koch et al. 2005). Η δεύτερη φάση καταλύεται από το ένζυμο ουριδινό-5- διφωσφογλυκουρο-μεταφοράση από το οποίο σχηματίζεται ο συζευγμένος μεταβολίτης.

Οι φθαλικοί εστέρες χαμηλού μοριακού βάρους συνήθως εκκρίνονται στα ούρα με τη μορφή του ελεύθερου μονοεστέρα ενώ οι μεγαλύτερου μοριακού βάρους αποβάλλονται κυρίως ή και αποκλειστικά συζευγμένοι. Στα ανθρώπινα ούρα το 70% του mEP που εκκρίνεται είναι σε ελεύθερη μορφή (Silva et al. 2005). Όσον αφορά στο μεταβολισμό του DnBP, το 6% του συνολικού mnBP αποβάλλεται ως έχει ενώ το υπόλοιπο με τη γλουκουρονιομένη μορφή του. Παρόμοια μεταβολικά μονοπάτια έχουν και τα DiBP και BBzP (Silva et al. 2005).

Το DEHP είναι ο πλέον χρησιμοποιούμενος φθαλικός εστέρας και επιπλέον είναι αυτό που έχει μελετηθεί περισσότερο. Εισερχόμενο στον οργανισμό μεταβολίζεται ταχύτατα ανεξαιρέτως τον τρόπο έκθεσης. Το πρώτο βήμα είναι η μετατροπή του DEHP στον αντίστοιχο μονοεστέρα, ο οποίος με τη σειρά του μεταβολίζεται ταχύτατα μέσω διάφορων αντιδράσεων οξείδωσης. Ο συνολικός μεταβολισμός του DEHP παρουσιάζεται στην Εικόνα 3 (Koch et al. 2004, Koch et al. 2005).
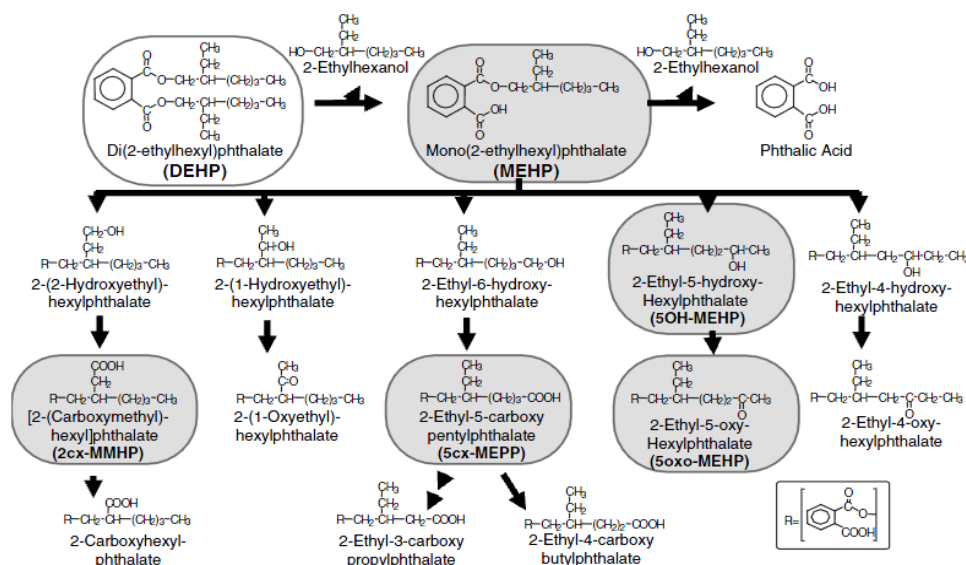
Εικόνα 3. Μεταβολισμός του DEHP. Οι κύριοι μεταβολίτες είναι τονισμένοι.

Το mEHP δεν σχηματίζεται μόνο από υδρόλυση του DEHP αλλά μπορεί να εισέλθει στον οργανισμό και από άλλες πηγές. Επίσης αντιπροσωπεύει μόνο το 10% του αρχικού DEHP, έχοντας και το μικρότερο χρόνο αποβολής από όλους τους υπόλοιπους μεταβολίτες. Οπότε, ο μεγάλος χρόνος αποβολής των mEOHP και mEHHP τα καθιστούν εξαίρετους βιοδείκτες επιβάρυνσης του οργανισμού.

Η ενδοκρινική δραστικότητα των PEs φέρεται να συνδέεται με μια σειρά αρνητικών επιπτώσεων στην ανθρώπινη υγεία. Μεταξύ άλλων, αναφέρονται συσχετίσεις με τη μείωση του αριθμού και της ποιότητας των σπερματοζωαρίων, όπως και βλάβες στο γενετικό υλικό του, μείωση των επιπέδων τεστοστερόνης στο αίμα, θανάτους εμβρύων, καρκινογενέσεις, δυσμορφίες, δυσλειτουργίες σε νεφρά και ήπαρ, κρυψορχίες, ενδομητριώσεις και πρόωρους τοκετούς (Hauser and Calafat 2005, Meeker et al. 2009).

<u>1.3. Δισφαινόλη-Α</u>

1.3.1 Ιδιότητες, χρήσεις και πηγές έκθεσης

       Η δομή της Δισφαινόλης-Α (Bisphenol-A, BPA) παρουσιάζεται στην Εικόνα 4. Η BPA είναι ένα λευκό στερεό με ήπια φαινολική οσμή. Έχει πολύ χαμηλή τάση ατμών και χαμηλό συντελεστή λιποφιλίας (logK$_{ow}$), υψηλό σημείο ζέσεως και μέτρια διαλυτότητα. Συντέθηκε αρχικά από τον A.P. Dianin, το 1891 και χρησιμοποιήθηκε αργότερα το 1930 σε μελέτες για συνθετικά οιστρογόνα.
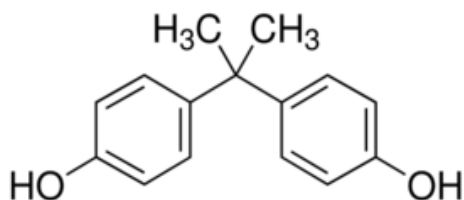
Εικόνα 4. Δομή BPA

       Παγκοσμίως, η BPA είναι ένα από τα ευρέως χρησιμοποιούμενα χημικά, με ετήσια παραγωγή που αγγίζει τους 3.000.000 τόνους, με 100 από αυτούς να απελευθερώνονται στην ατμόσφαιρα (Flint et al. 2012, Vandenberg et al. 2007). Η BPA, χρησιμοποιείται στην παραγωγή πολυανθρακικών πλαστικών, ρητινών, επιβραδυντικών φλόγας και φυτοφαρμάκων. Κατά συνέπεια μπορεί να βρεθεί σε πάρα πολλά προϊόντα όπως: πλαστικά, οδοντικά σφραγίσματα, προϊόντα προσωπικής περιποίησης, δομικά υλικά, κονσέρβες, φακούς επαφής, ηλεκτρονικές συσκευές, θερμικό χαρτί (αποδείξεων) κλπ..

1.3.2. Μεταβολισμός και επιδράσεις στην υγεία

       Η ανθρώπινη έκθεση στη BPA, γίνεται κυρίως δια μέσου της εισπνοής, της δερματικής επαφής και της κατάποσης. Απορροφάται πλήρως από τη γαστρεντερική οδό και στη συνέχεια βιομετατρέπεται από τα ένζυμα του γαστρεντερικού συστήματος και του ήπατος. Αποβάλλεται μέσω των ούρων κυρίως στη γλουκουρονιδιομένη και δευτερευόντως στη σουλφονιωμένη και ελεύθερη μορφή της ή με την σουλφονιωμένη μορφή (Εικόνα 5).

       Παρατηρώντας την δομή της BPA και συγκρίνοντας την με αυτήν της εστραδιόλης (Εικόνα 6), η οποία αποτελεί φυσική ορμόνη, εξηγείται η ενδοκρινική της δράση (Chouhan et al. 2014, Peretz et al. 2014, Rochester 2013). Η έκθεση στη BPA έχει συνδεθεί με καρδιακές ασθένειες, ανωμαλίες στο συκώτι, μεταβολές στη λειτουργία του θυρεοειδή αδένα, αναπαραγωγικά προβλήματα, άσθμα, άγχος, παχυσαρκία, διαβήτη, καρκίνο του μαστού και προστάτη.
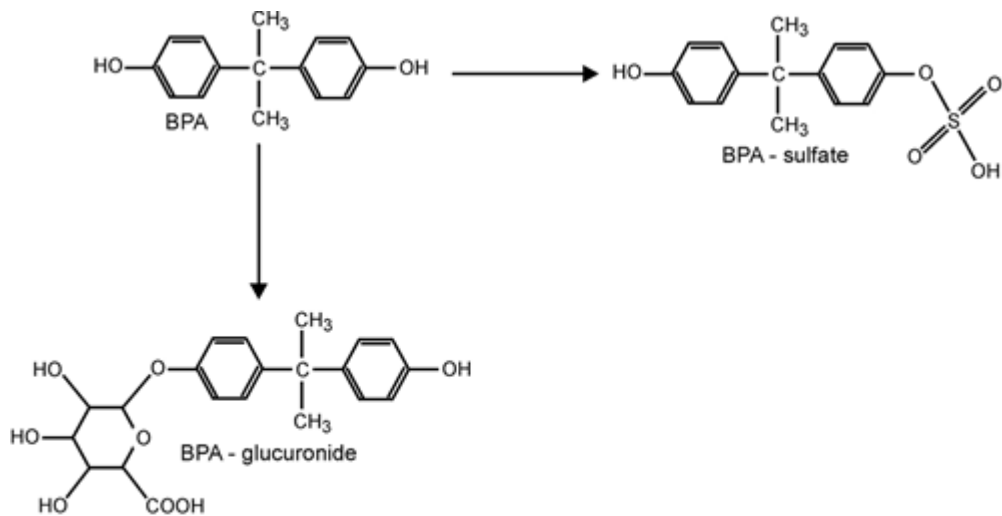
Εικόνα 5. Μεταβολίτες της BPA
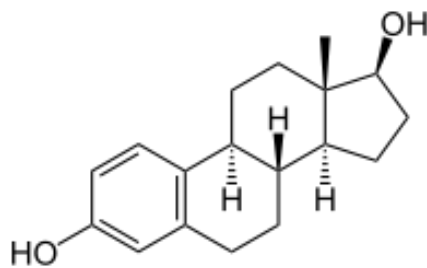
Εικόνα 6. Δομή εστραδιόλης

1.4. Παραβένια

1.4.1 Ιδιότητες, χρήσεις και πηγές έκθεσης

      Τα παραβένια αποτελούν μια ομόλογη σειρά εστέρων του π-υδρόξυ-βενζοϊκού οξέος. Η γενική δομή τους φαίνεται στην Εικόνα 7. Τα PBs είναι σταθερά σε όλη την κλίμακα του pH και επαρκώς διαλυτά στο νερό. Σε καθαρή μορφή είναι μικροί, άχρωμοι κρύσταλλοι χωρίς γεύση ή οσμή. Η τάση λιποφιλίας στα PBs, τείνει να αυξάνεται όσο αυξάνεται και η αλκυλική αλυσίδα τους. Γενικά είναι σταθερά στον αέρα και δεν υδρολύονται σε όξινα διαλύματα. Τα μικρότερης αλυσίδας PBs διαλύονται στο νερό, ενώ τα μεγαλύτερης σε οργανικούς διαλύτες (Darbre and Harvey 2008).
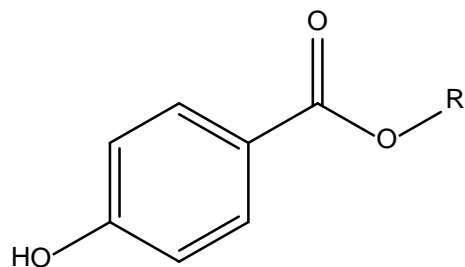
Εικόνα 7. Γενική δομή PBs

Τα PBs χρησιμοποιούνται ως αντιμικροβιακά συντηρητικά, ειδικότερα κατά της μούχλας και των ζυμών σε καλλυντικά και φαρμακευτικά προϊόντα και στην επεξεργασία ποτών και τροφίμων (Elder 1984). Μεμονωμένα ή σε συνδυασμούς, τα PBs χρησιμοποιούνται σε πάνω από 13.200 σκευάσματα. Η αντιμικροβιακή τους δράση αυξάνεται, όσο το μήκος της αλκυλικής αλυσίδας αυξάνεται (Golden et al., 2005). Χρησιμοποιούνται ως συντηρητικά στα καλλυντικά, γιατί χαρακτηρίζονται από χαμηλή τοξικότητα, αδράνεια, εύκολη βιοαποικοδόμηση, χαμηλό κόστος και μεγάλο εύρος εφαρμογών.

Τα PBs προστίθενται στα τρόφιμα για πάνω από πενήντα χρόνια. Χρησιμοποιούνται στην επεξεργασία λαχανικών, στα λίπη και στα έλαια, στα υποκατάστατα ζάχαρης, στους φρουτοχυμούς, στις σάλτσες, στα κατεψυγμένα γαλακτοκομικά προϊόντα, σε συγκεντρώσεις μεταξύ 450-2000 ppm (Soni et al. 2001a, b, Soni et al. 2005). Το μέθυλο-παραβένιο (methyl paraben, MPB) υπάρχει φυσικά στο λευκό κρασί, στα βατόμουρα και σε κάποια φρούτα του δάσους. Επίσης, το κ-πρόπυλο-παραβένιο (n-propyl paraben, nPPB) ανιχνεύθηκε στο φυτό *Stocksia brahuic.*

Επίσης χρησιμοποιούνται ευρύτατα στα καλλυντικά και στα φαρμακευτικά σκευάσματα σκευασμάτων συμπεριλαμβανομένων αναισθητικών, χαπιών, σιροπιών, ενέσιμων διαλυμάτων. Όπως έχει προαναφερθεί, η έκθεση στον άνθρωπο γίνεται μέσω κατάποσης, εισπνοής και δερματικής επαφής αλλά το μεγαλύτερο ποσοστό της ανθρώπινης έκθεσης στα PBs πραγματοποιείται μέσω της δερματικής επαφής κατά την χρήση καλλυντικών σκευασμάτων (Darbre and Harvey 2008, Soni et al. 2001a, b, Soni et al. 2005).

1.4.2. Μεταβολισμός και επιδράσεις στην υγεία

Γενικά, τα PBs απορροφούνται πολύ καλά από το δέρμα και ο εστερικός δεσμός τους υδρολύεται από ένζυμα που ονομάζονται καρβοξυλεστεράσες. Η απορρόφηση τους εξαρτάται από το μήκος της αλκυλικής αλυσίδας και μειώνεται με την αύξηση του μήκους της. Είτε μεταβολίζονται στο πάρα-ύδροξυ-βενζοϊκό οξύ είτε σχηματίζουν γλουκουρονικά, θειικά και γλυκινικά συζεύγματα. Τέλος, αποβάλλονται από τον ανθρώπινο οργανισμό κυρίως μέσω των ούρων (Darbre and Harvey 2008). Στην Εικόνα 8 φαίνονται και οι κύριοι μεταβολίτες των PBs.
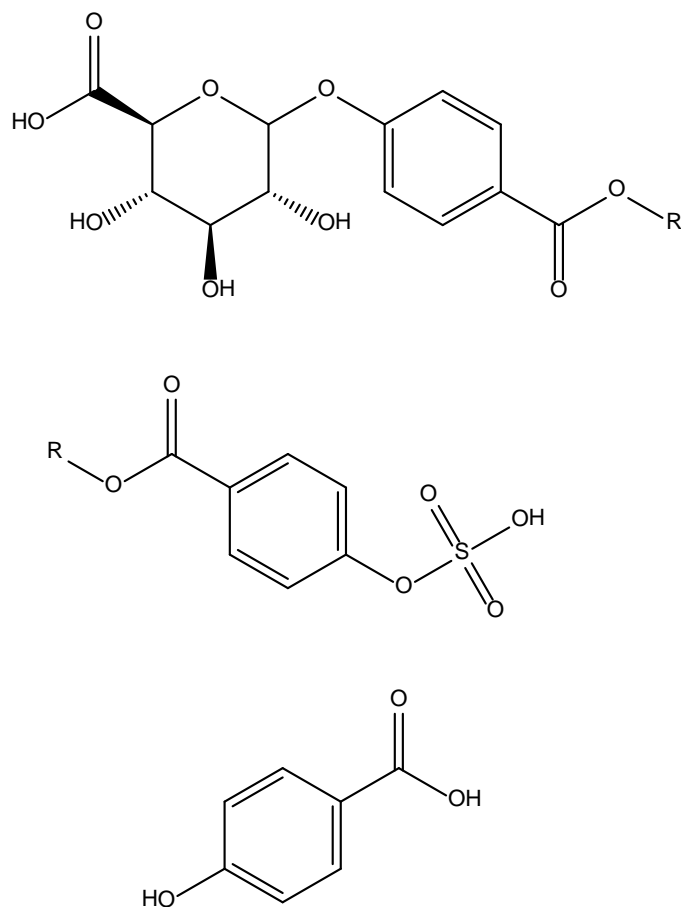
Εικόνα 8. Κύριοι μεταβολίτες των PBs: α) γλουκουρονιδιωμένο σύζευγμα, β) σουλφονιωμένο σύζευγμα, γ) π-υδρόξυ-βενζοϊκό οξύ

Έχει παρατηρηθεί ότι προκαλούν την *in vitro* αύξηση των MCF-7 ανθρώπινων καρκινικών κυττάρων και γενικότερα έχουν συσχετιστεί με την εμφάνιση καρκίνου του μαστού. Έχει αναφερθεί ότι προκαλούν δερματικές αντιδράσεις και αλλεργίες ενώ επηρεάζουν τα επίπεδα οιστρογόνων, μέσω της αναστολής της δράσης της σουλφοτρανσφεράσης μέσα στο κυτοσόλιο των ανθρώπινων δερματικών κυττάρων (Crinnion 2010, Darbre and Harvey 2008, Soni et al. 2001a, Soni et al. 2005).

<u>1.5. Αναλυτικές τεχνικές προσδιορισμού σε ανθρώπινα ούρα</u>

Οι αναλυτικές μέθοδοι που έχουν αναφερθεί μέχρι τώρα στη βιβλιογραφία, για τον προσδιορισμό των μεταβολιτών των PEs, της BPA και των PBs , χρησιμοποιούν κυρίως υγρή χρωματογραφία υψηλής απόδοσης συζευγμένη με διαδοχική φασματομετρία μάζας (High Performance Liquid Chromatography-Tandem Mass Spectrometry, HPLC-MS/MS) με πηγή ιοντισμού είτε ηλεκτροψεκασμό (electrospray ionization, ESI), είτε χημικό ιονισμό ατμοσφαιρικής πίεσης (atmospheric pressure chemical ionization, APCI) (Dewalque et al. 2014, Nicolucci et al. 2013, Silva et al. 2003, Silva et al. 2004) αν και δεν υπάρχει καμία αναφορά για ταυτόχρονη ανάλυση των παραπάνω ενώσεων. Επίσης, ο διαχωρισμός των ισομερών κ- και ίσο- των

βούτυλο-παραβενίου (butyl paraben, BPB) και PPB έχει επιτευχθεί μόνο με τη χρήση υγρής χρωματογραφίας υπερ-υψηλής απόδοσης (Ultra Performance Liquid Chromatography, UPLC) (MS 2011).

Η πολικότητα των προς μελέτη ενώσεων, η υδατική σύσταση των ούρων όπως και η περιπλοκότητα τους σαν μήτρα και τέλος η ανάγκη για πολύ χαμηλά όρια ανίχνευσης και αναλύσεις μεγάλων αριθμών δειγμάτων για να καλυφθούν οι ανάγκες των επιδημιολογικών μελετών πρακτικά καθιστούν μονόδρομο την επιλογή HPLC-MS/MS σαν αναλυτική μεθοδολογία. Συνοπτικά, οι περισσότερες αναλυτικές μεθοδολογίες αποτελούνται από τα ακόλουθα δύο στάδια επεξεργασίας:

Α) Ενζυμική υδρόλυση των συζευγμένων μεταβολιτών είτε με τη χρήση του ενζύμου E.coli β-glucuronidase για την μέτρηση των ελεύθερων και γλουκουρονιδιομένων μεταβολιτών είτε του ενζύμου H.Pomatia β-glucuronidase, για την μέτρηση των ελεύθερων, των γλουκουρονιδιομένων και των σουλφονιωμένων μεταβολιτών των ενώσεων (Dewalque et al. 2014).

Β) Καθαρισμός των δειγμάτων ούρων με εκχύλιση στερεάς φάσης (SPE), είτε συμβατική (Dewalque et al. 2014) είτε αυτόματη (Silva et al. 2004) είτε ακόμα και σε σειρά με το HPLC-MS/MS (Ye et al. 2005).

1.6. Μελέτη «ΡΕΑ»

Η υπόθεση ότι τα εμβρυϊκά και πρώιμα γεγονότα της ζωής οδηγούν σε μόνιμες μεταβολικές ή αναπτυξιακές αλλαγές από την δεκαετία του '80 ακόμα, μετά από μια σειρά σημαντικών επιδημιολογικών παρατηρήσεων που συνέδεσαν την μείωση του βάρους γέννησης με έναν αυξημένο κίνδυνο ασθενειών της ενήλικης ζωής, συμπεριλαμβανομένου του σακχαρώδους διαβήτη τύπου-2, του μεταβολικού συνδρόμου, της υπέρτασης, και των καρδιαγγειακών παθήσεων (World Health Organization 2012).

Για την προώθηση της γνώσης στον τομέα αυτό απαιτούνται μετρήσεις οι οποίες να είναι ακριβείς και να μπορούν να αναδείξουν τις αιτιολογικές σχέσεις μεταξύ έκθεσης και ασθένειας, αλλά και να επιτρέπουν την διατύπωση συστάσεων και την διαμόρφωση πολιτικών δημόσιας υγείας που να βελτιώνουν το περιβάλλον ανάπτυξης των παιδιών και να μειώνουν το κοινωνικοοικονομικό κόστος σοβαρών χρόνιων ασθενειών. Ο καταλληλότερος σχεδιασμός τέτοιων μελετών είναι η προοπτική επιδημιολογική μελέτη μητέρας-παιδιού (mother-child cohort), στα πλαίσια της οποίας τόσο οι περιβαλλοντικές εκθέσεις όσο και η κατάσταση υγείας των παιδιών παρακολουθούνται από την φάση της κύησης μέχρι και την ενηλικίωση και αναζητούνται συσχετισμοί μεταξύ τους. Στην Ευρώπη έχουν πραγματοποιηθεί αρκετές προοπτικές μελέτες μητέρας-παιδιού κατά τις τελευταίες δεκαετίες.

Η μελέτη Μητέρας-Παιδιού Κρήτης (Μελέτη ΡΕΑ), είναι η πρώτη και μοναδική μελέτη μητέρας-παιδιού που πραγματοποιείται στην Ελλάδα. Περιλαμβάνει ένα δείγμα περίπου 1.500 εγκύων γυναικών (Ελληνίδων και αλλοδαπών) οι οποίες έμειναν έγκυε κατά το έτος 2008 και των παιδιών τους πλέον, στο νομό Ηρακλείου (Chatzi et al. 2009, Patelarou et al. 2011).

1.7. Στόχος της διατριβής

Ο κύριος στόχος της παρούσας διατριβής ήταν η εκτίμηση της έκθεσης σε PEs, PBs και BPA κατά την εμβρυϊκή και πρώιμη παιδική ηλικία για πρώτη φορά στην Ελλάδα. Αρχικά, η μελέτη εστιάστηκε στην ανάπτυξη μιας αναλυτικής μεθόδου προσδιορισμού των μεταβολιτών των προαναφερθέντων ενδοκρινικών διαταρακτών σε ανθρώπινα ούρα με χρήση HPLC-MS/MS. Τα επιθυμητά χαρακτηριστικά της αναλυτικής διαδικασίας ήταν τα εξής:

I) ο ταυτόχρονος προσδιορισμός των μεταβολιτών των PEs, PBs και BPA
II) ο χρωματογραφικός διαχωρισμός των ισο- και κ- ισομερών των PPB και BPB με χρήση συμβατικών χρωματογραφικών στηλών και αντλιών υψηλής απόδοσης (χωρίς τη χρήση UPLC)
III) την επίτευξη των ελαχίστων δυνατών ορίων ανίχνευσης και ποσοτικοποίησης
IV) και τέλος την ικανότητα ανάλυσης μεγάλου αριθμού δειγμάτων

Η μέθοδος που αναπτύξαμε εφαρμόστηκε στην ανάλυση δειγμάτων ούρων προερχόμενων από συμμετέχοντες στη μελέτη «PEA» και πιο συγκεκριμένα από διακόσιες τριάντα εννέα (239) εγκυμονούσες γυναίκες, από τα διακόσια τριάντα εννέα (239) παιδιά τους στην ηλικία των 2.5 ετών και από πεντακόσια (500) παιδιά 4 ετών. Οι επιμέρους στόχοι για την εκτίμηση της έκθεσης ήταν οι παρακάτω:

V) ο προσδιορισμός για πρώτη φορά στην Ελλάδα των επιπέδων μεταβολιτών των PEs, των PBs και της BPA,
VI) η διερεύνηση της σχέσης της έκθεσης πριν (εγκυμοσύνη) και μετά τη γέννηση (2.5 και 4 έτη),
VII) η εκτίμηση της ημερήσιας πρόσληψης σε PEs, PBs και BPA
VIII) η εκτίμηση των πηγών έκθεσης
IX) η σύγκριση των επιπέδων έκθεσης στην Ελλάδα με άλλες αντίστοιχες μελέτες μέσω συστηματικής βιβλιογραφικής αναζήτησης

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2. Αποτελέσματα και συζήτηση

2.1. Επισκόπηση κυρίων αποτελεσμάτων

Ο αρχικός σκοπός της παρούσας μελέτης ήταν η ανάπτυξη μιας νέας αναλυτικής μεθόδου ώστε να επιτευχθεί ο ταυτόχρονος προσδιορισμός των μεταβολιτών των PEs, των PBs και της BPA σε ανθρώπινα ούρα. Η μεθοδολογία που αναπτύχθηκε περιλαμβάνει τρία κύρια στάδια: την ενζυμική υδρόλυση των συζευγμένων μορφών των ενώσεων προς μελέτη στις αντίστοιχες ελεύθερες, τον καθαρισμό των δειγμάτων ούρων με SPE και την ανάλυση με HPLC-MS/MS.

Η ενζυμική υδρόλυση των γλουκουρονιδιομένων και σουλφονιωμένων μορφών των μεταβολιτών στα ανθρώπινα ούρα είναι απαραίτητη ώστε να προσδιοριστούν τα συνολικά επίπεδα ενός μεταβολίτη μετρώντας μόνο την ελεύθερη μορφή του. Οι υδρολύσεις τέτοιου τύπου έχουν μελετηθεί στο παρελθόν και προσαρμόστηκαν στις ανάγκες της παρούσας μελέτης.

Στη συνέχεια, δόθηκε έμφαση στην βελτιστοποίηση ενός πρωτόκολλου επεξεργασίας καθαρισμού των δειγμάτων ούρων που να πληροί τις εξής προυποθέσεις: ικανοποιητική ανάκτηση και των τριών κατηγοριών ενδοκρινικών διαταρρακτών, επαρκή καθαρισμό των δειγμάτων ούρων και ικανότητα ταυτόχρονης ανάλυσης του μέγιστου αριθμού δειγμάτων με τον διαθέσιμο εξοπλισμό του ΕΠΕΧΗΔΙ. Η τελευταία προϋπόθεση επέβαλλε την επιλογή της SPE λόγω της σύντομης επεξεργασίας, την εμπλοκή αναλωσίμων μιας χρήσεως (σωλήνες falcon, φυσίγγια SPE) και την ταυτόχρονη ανάλυση πολλών δειγμάτων στο ίδιο SPE manifold (συσκευή όπου συνδέονται 12 ή και περισσότερα φυσίγια SPE και εφαρμόζεται κενό για τη ρύθμιση της ροής). Το πρωτόκολλο που αναπτύχθηκε με χρήση προενεργοποιημένων cartidge Agilent Nexus, κατακράτησε αποτελεσματικά τους μεταβολίτες των PEs, τα PBs και την BPA ενώ τα δείγματα καθαρίστηκαν αποτελεσματικά, κάτι που δεν είχε αναφερθεί μέχρι τώρα στη βιβλιογραφία. Όσον αφορά στη χρωματογραφική ανάλυση, αφενός ο γενικός στόχος ήταν η ταυτόχρονη ανάλυση των μεταβολιτών των PEs, των PBs και της BPA και αφετέρου ο διαχωρισμός των ισο- και κ- ισομερών των PPB και BPB, με χρήση συμβατικών χρωματογραφικών στηλών και αντλιών.

Τα συγκεκριμένα ισομερή είχαν διαχωριστεί στο παρελθόν μόνο με χρήση UPLC. Το κύριο πρόβλημα με αυτά τα δύο ισομερή είναι ότι δίνουν ακριβώς τα ίδια ιόντα στο φασματογράφο μάζας και κατά συνέπεια ο διαχωρισμός τους βασίζεται αποκλειστικά στη χρωματογραφία. Τα τελικά αποτελέσματα ήταν ιδιαίτερα ικανοποιητικά όπως φαίνεται χαρακτηριστικά στην Εικόνα 9 όπου παρατίθεται χρωματογράφημα δείγματος ούρων, εμβολιασμένο με 100 ng όλων των προς μελέτη ενώσεων.
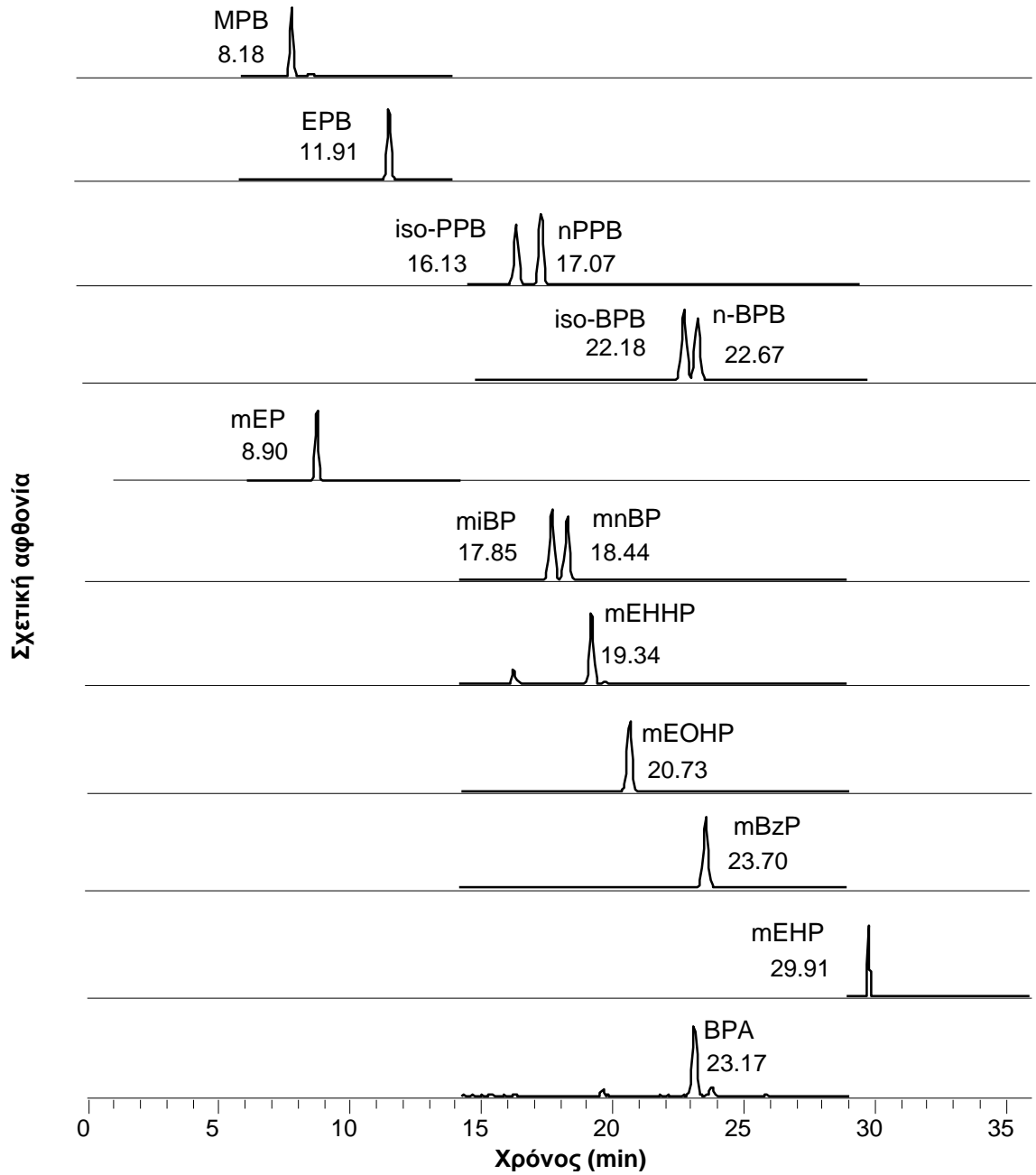
Εικόνα 9. Χαρακτηριστικό χρωματογράφημα δείγματος ούρων όπου παρακολουθούνται ταυτόχρονα οι μεταβολίτες των PEs, τα PBs και η BPA

Παρόλα αυτά, τα πολύ χαμηλά επίπεδα της BPA στα ανθρώπινα ούρα και η ως ένα βαθμό περιορισμένη ευαισθησία του διαθέσιμου φασματογράφου μάζας σε σχέση με αντίστοιχα νεότερα μοντέλα οδήγησε στην προσθήκη ενός ακόμα σταδίου στην αναλυτική μέθοδο. Μετά την ολοκλήρωση της χρωματογραφικής ανάλυσης για μεταβολίτες των PEs και PBs, η BPA παραγοντοποιούνταν με την προσθήκη

dansyl chloride σε βασικό pH και θέρμανση. Η αντίδραση πραγματοποιούνταν μέσα στο φιαλίδιο του αυτόματου δειγματολήπτη και δεν απαιτούνταν περαιτέρω επεξεργασία πριν την ανάλυση στο HPLC-MS. Η παραγοντοποίηση βελτίωσε θεαματικά τα όρια ανίχνευσης της μεθόδου (2.01ng/mL - 0.007 ng/mL). Ένα χρωματογράφημα πραγματικού δείγματος όπου παρακολουθείται η παραγοντοποιημένη BPA παρατίθεται στην Εικόνα 10.
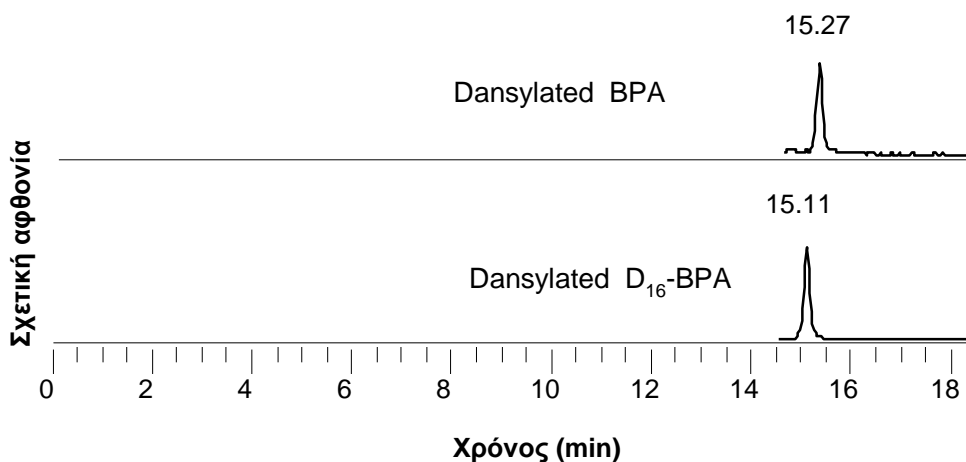
Εικόνα 10. Χρωματογράφημα δείγματος ούρων όπου παρακολουθείται η dansylated-BPA και το δευτεριωμένο ανάλογο της

Η προαναφερθείσα μέθοδος εφαρμόστηκε με επιτυχία στην ανάλυση εννιακοσίων εβδομήντα οκτώ (978) δειγμάτων ούρων προερχόμενα από τρεις πληθυσμούς της μελέτης «PEA» για τον προσδιορισμό των επιπέδων συγκεντρώσεων επτά μεταβολιτών των PEs, έξι PBs και της BPA. Τα δείγματα αποτελούνταν από διακόσια τριάντα εννέα (239) ζευγάρια μητέρας (4ος μήνας εγκυμοσύνης) - παιδιού (2.5 έτη) και από πεντακόσια (500) παιδιά προσχολικής ηλικίας. Η μελέτη εστιάστηκε στους παραπάνω πληθυσμούς επειδή οι βλαπτικές επιδράσεις των ενδοκρινικών διαταρακτών είναι εντονότερες κατά την εμβρυϊκή ζωή και την πρώιμη παιδική ηλικία.

Αξίζει να σημειωθεί ότι τα επίπεδα έκθεσης σε PEs, PBs και BPA εκτιμήθηκαν για πρώτη φορά στην Ελλάδα και σε συνδυασμό με την ύπαρξη δεδομένων από τρεις πληθυσμούς σε αυτό τον αριθμό δειγμάτων, αποτελούν σημαντική συνεισφορά στη διεθνή βιβλιογραφία.

Τα επίπεδα συγκεντρώσεων των μεταβολιτών των PEs και της BPA ήταν σε συγκρίσιμα επίπεδα με αντίστοιχες εργασίες από άλλες χώρες και στις τρεις πληθυσμιακές ομάδες. Τα επίπεδα συγκεντρώσεων των PBs και στους δυο πληθυσμούς των παιδιών βρέθηκαν σε υψηλότερα επίπεδα σε σχέση με μια αντίστοιχη εργασία που έγινε στη Δανία. Ωστόσο, οι διαφοροποιήσεις μεταξύ των μελετών (χρόνος δειγματοληψίας, ηλικία παιδιών) και η μη ύπαρξη περισσότερων σχετικών αναφορών καθιστά δύσκολη την εξαγωγή ασφαλών συμπερασμάτων.

Σε τρεις PEs (DEP, DnBP και BBP) και στο EPB βρέθηκαν ασθενείς αλλά στατιστικά σημαντικές συσχετίσεις (συντελεστής συσχέτισης, CC: 0.1-0.2, p<0.01) μεταξύ των επιπέδων στις μητέρες κατά την εγκυμοσύνη και στα παιδιά τους περίπου τρία χρόνια αργότερα (Πίνακας 1). Συνυπολογίζοντας ότι ο χρόνος αποβολής αυτών των ενώσεων από ανθρώπινο οργανισμό είναι της τάξης λίγων ωρών και άρα αποκλείοντας το ενδεχόμενο βιοσυσσώρευσης, διαφαίνεται η ύπαρξη κοινών και σταθερών πηγών εκπομπής (σπίτι, αυτοκίνητο κλπ.) στη διάρκεια του χρόνου. Η ακριβής ηλικία των παιδιών συσχετίστηκε αρνητικά με τα επίπεδα συγκεντρώσεων όλων των μελετώμενων ενώσεων στον πληθυσμό των 2.5 ετών, κάτι το οποίο δεν παρατηρήθηκε στα παιδιά προσχολικής ηλικίας. Το παραπάνω εξηγείται με τη χαρακτηριστική τάση των παιδιών να βάζουν διάφορα αντικείμενα στο στόμα τους (floor-to-mouth behaviour), η οποία ατονεί όσο μεγαλώνουν και με το μεγαλύτερο λόγο μάζας προσλαμβανόμενης τροφής/μάζα σώματος (έκθεση μέσω της τροφής) και επιφάνειας δέρματος/μάζα σώματος (έκθεση μέσω της δερματικής επαφής) στις μικρότερες ηλικίες.

Με χρήση μοντέλων τοξικοκινητικής, υπολογίστηκε η ημερήσια έκθεση πρόσληψη ανά kg σωματικού βάρους. Τα παιδιά βρέθηκαν να έχουν είχαν χαμηλότερα επίπεδα πρόσληψης σε σχέση με τις εγκυμονούσες ημέρες σε όλες τις μελετώμενες ενώσεις εκτός από το DEHP και την BPA. Επίσης, τα επίπεδα στα παιδιά 4 ετών είχαν χαμηλότερα επίπεδα ημερήσιας πρόσληψης σε σχέση με τα παιδιά 2.5 ετών (Εικόνα 11).

Πίνακας 1. Συντελεστές συσχέτισης (2-tailed Spearman, p< 0.01) μεταξύ των επιπέδων συγκεντρώσεων στις εγκυμονούσες γυναίκες, τα παιδιά και την ηλικία των παιδιών.

	C-DEP	C-DiBP	C-DnBP	C-BBzP	C-DEHP	C-MPB	C-EPB	C-nPPB	C-BPA
M-DEP	0.20								
M-DiBP									
M-DnBP			0.23						
M-BBzP				0.18					
M-MPB									
M-EPB							0.13		
M-BPA									
C-Ηλικία (y)	-0.43	-0.43	-0.48	-0.37	-0.19	-0.38	-0.47	-0.38	-0.21

M-: εγκυμονούσες γυναίκες, C-: παιδιά

Σε όλες τις πληθυσμιακές κατηγορίες, βρέθηκαν ορισμένες περιπτώσεις που υπερέβαιναν τα θεσμισμένα ανώτατα όρια της Αμερικάνικης Υπηρεσίας Προστασίας Περιβάλλοντος (U.S. Environmental Protection Agency Tolerable Daily Intake, USEPA-TDI) όπως και της Ευρωπαϊκής Αρχής Ασφάλειας Τροφίμων (European Food Safety Authority Reference Doses, EFSA-RfD). Για παράδειγμα, το 3.6% των 4-χρονων παιδιών υπερέβη το όριο της EFSA για το DEHP.

Με βάση περιβαλλοντικές μετρήσεις επιπέδων PEs σε αέρα εσωτερικού χώρου οικιών, αυτοκινήτων και πόσιμου νερού στην περιοχή του Ηρακλείου, υπολογίστηκε η ημερήσια πρόσληψη μέσω της αναπνοής

και της κατανάλωσης πόσιμου νερού. Η συνεισφορά και των δυο αυτών μονοπατιών έκθεσης βρέθηκε να είναι αρκετά χαμηλή σε σχέση με την συνολική ημερήσια πρόσληψη που υπολογίστηκε με βάση τις συγκεντρώσεις των μεταβολιτών στα ούρα.
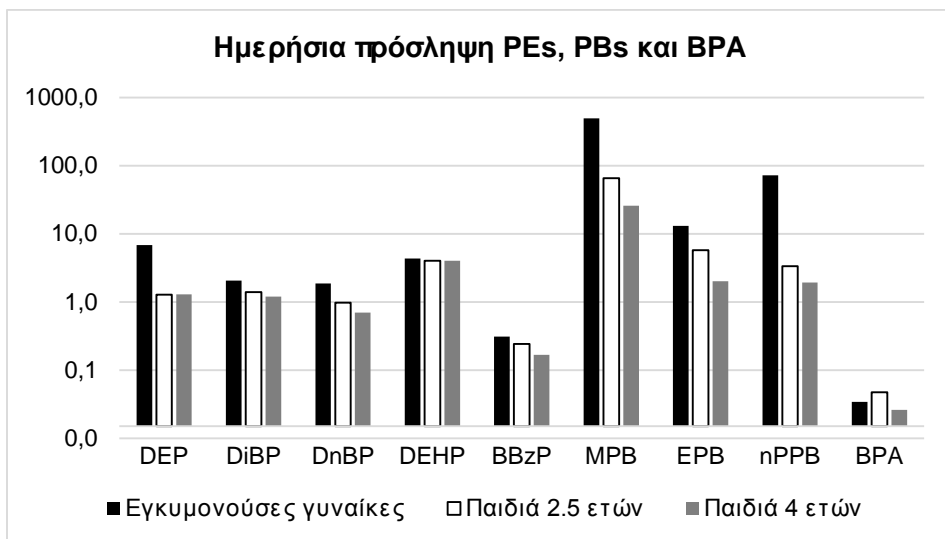
Εικόνα 11. Σύγκριση επιπέδων ημερήσιας πρόσληψης (διάμεσες τιμές, μg x kg^{-1} x d^{-1})

Οι πηγές έκθεσης διερευνήθηκαν με ανάλυση κυρίων συνιστωσών (Principal component analysis, PCA), η οποία ομαδοποίησε την έκθεση σε δυο κύριες κατηγορίες ανά ομάδα πληθυσμού. Χαρακτηριστικά παρατίθενται τα αποτελέσματα της ανάλυσης στα ζευγάρια μητέρας-παιδιού στον Πίνακα 2. Η έκθεση φαίνεται να γίνεται σε μίγματα των ενώσεων και πιο συγκεκριμένα σε μίγματα PEs και BPA (παράγοντας 1 - παιδιά, παρ. 2 - εγκυμονούσες, Πίνακας 2), όπου χρησιμοποιούνται κατά κόρον στο πλαστικό και σε μίγματα PBs και DEP (παρ. 3 - παιδιά, παρ. 4 -   εγκυμονούσες), που χρησιμοποιούνται ευρύτατα στα προϊόντα προσωπικής φροντίδας-υγιεινής. Η ίδια εικόνα παρουσιάστηκε και στα 4-χρονα παιδιά.

Τέλος, μελετήθηκε ο ρυθμός μεταβολισμού του DEHP, υπολογίζοντας τους σχετικούς μεταβολικούς ρυθμούς (ταχύτητα μετατροπής των μεταβολιτών σε δευτερεύοντες μεταβολίτες). Αναλυτικότερα, υπολογίστηκαν οι σχετικοί μεταβολικοί ρυθμοί (RMR), οι οποίοι εκφράζουν την ταχύτητα μετατροπής του mEHP στο mEHHP (RMR$_1$) και του mEHHP στο mEOHP (RMR$_2$). Βρέθηκαν στατιστικά σημαντικές (p<0.01) διαφοροποιήσεις στις ταχύτητες μετατροπής ανάμεσα στις εγκυμονούσες και τα παιδιά όπως και ανάμεσα στα αρσενικά και θηλυκά παιδιά. Στην Εικόνα 12 φαίνεται χαρακτηριστικά η διαφορά στα επίπεδα του RMR$_1$ ανάμεσα στα παιδιά 2.5 και 4 ετών.

Πίνακας 2. Παράγοντες ανάλυσης κυρίων συνιστωσών

Παράγοντας	1	2	3	4
C-DnBP	0.897			
C-DiBP	0.880			
C-DEHP	0.843			
C-BBzP	0.803			
C-DEP	0.662		0.319	
C-MPB			0.899	
C-EPB			0.856	
C-nPPB			0.906	
C-BPA	0.635			
M-DnBP		0.752		
M-DiBP		0.748		
M-DEHP		0.844		
M-BBzP		0.790		
M-DEP		0.448		0.421
M-MPB				0.913
M-EPB				0.740
M-nPPB				0.906
M-BPA		0.509		

Συντελεστές <0.200 δεν παρουσιάζονται, M-: εγκυμονούσες γυναίκες, C-: παιδιά
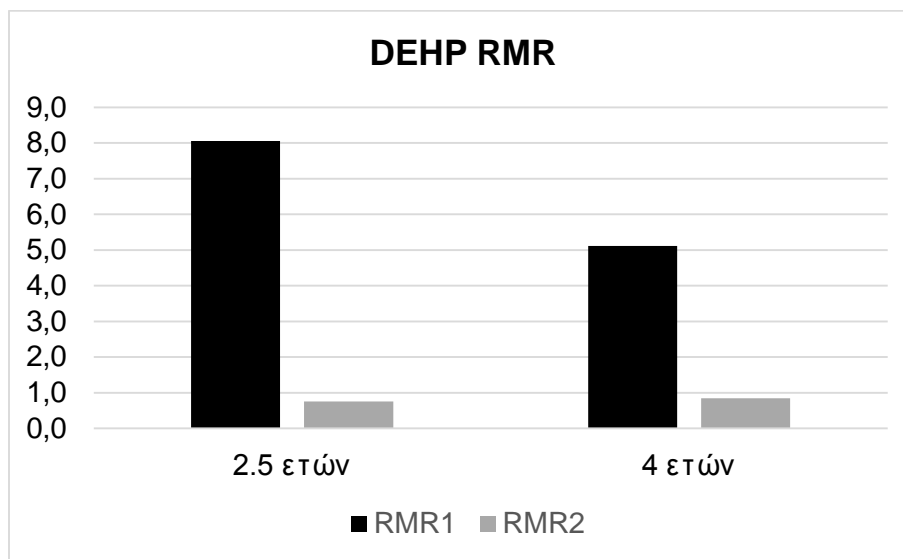
Εικόνα 12. Σύγκριση RMR στα παιδιά 2.5 και 4 ετών, μέσες τιμές

2.2. Παρουσίαση αποτελεσμάτων των επιμέρους Εργασιών

Τα αποτελέσματα της Διατριβής κατανεμήθηκαν σε τρείς διαφορετικές Εργασίες προς δημοσίευση. Πιο συγκεκριμένα:

Εργασία #1: Η Εργασία αυτή περιέχει την ανάπτυξη όλης της αναλυτικής διαδικασίας, τα αποτελέσματα και τις επιδόσεις της μεθόδου, και την εφαρμογή της στην ανάλυση πραγματικών δειγμάτων.

Εργασία #2: Στην δεύτερη Εργασία γίνεται αναφορά στον προσδιορισμό των επιπέδων των PEs, BPA και PBs σε 239 ζεύγη μητέρας-παιδιού της κοόρτης "PEA". Ακολούθως παρουσιάζεται λεπτομερής στατιστική επεξεργασία με στόχο την ερμηνεία των αποτελεσμάτων για την αποτίμηση της έκθεσης των συγκεκριμένων πληθυσμιακών ομάδων στους υπό μελέτη ενδοκρινικούς διαταράκτες, τον εντοπισμό των κύριων πηγών έκθεσης τον υπολογισμό των σχετικών ρυθμών μεταβολισμού του DEHP και τη συστηματική σύγκριση με άλλες μελέτες σε χώρες της Ευρώπης, της Ασίας και των Η.Π.Α..

Εργασία #3: Στη τρίτη Εργασία παρουσιάζονται τα αποτελέσματα της μελέτης της έκθεσης στους προαναφερόμενους ενδοκρινικούς διαταράκτες σε ένα πληθυσμό πεντακοσίων (500) παιδιών προσχολικής ηλικίας, επίσης μέλη της κοόρτης «Ρέα». Επίσης και στην Εργασία#3 παρουσιάζεται λεπτομερής στατιστική επεξεργασία με στόχο την ερμηνεία των αποτελεσμάτων για την αποτίμηση της έκθεσης της συγκεκριμένης πληθυσμιακής ομάδας στις υπό μελέτη ουσίες, στον εντοπισμό των κύριων πηγών έκθεσης με αναφορά και σε περιβαλλοντικές αναλύσεις (ατμόσφαιρα, πόσιμο νερό), τη συσχέτιση με τα δεδομένα από τα παιδιά στα 2.5 έτη (Εργασία #2) και τη συστηματική σύγκριση με άλλες μελέτες σε χώρες της Ευρώπης, της Ασίας και των Η.Π.Α.. Τέλος στην εν λόγω Εργασία προσεγγίζουμε και τον μεταβολισμό του DEHP σε σχέση με την ηλικία των παιδιών.

2.2.1. Εργασία #1

Αναπτύχθηκε μια νέα μέθοδος προσδιορισμού των επιπέδων επτά μεταβολιτών των PEs, έξι PBs και της BPA σε ανθρώπινα ούρα με χρήση HPLC-MS/MS. Οι τρεις αυτές κατηγορίες ενδοκρινικών διαταρακτών απομονώθηκαν με εκχύλιση στερεάς φάσης (solid phase extraction, SPE) για πρώτη φορά ταυτόχρονα από τα δείγματα ούρων. Επιπλέον για πρώτη φορά αναφέρεται ο χρωματογραφικός διαχωρισμός των ισο- και κ- ισομερών των PPB και BPB χωρίς τη χρήση χρωματογραφίας UPLC (με συμβατικές στήλες ανάστροφης φάσης).

Η ανάλυση στο HPLC-MS/MS βελτιστοποιήθηκε με δύο πηγές ιονισμού, ESI APCI. Επιλέχθηκε ο ESI για την καλύτερη σταθερότητα και επαναληψιμότητα που επέδειξε. Τα όρια ανίχνευσης της μεθόδου (method limits of detection, mLOD) την ταυτόχρονη χρωματογραφική ανάλυση και των τριών κατηγοριών ενώσεων κυμάνθηκαν μεταξύ 0.01 και 0.84 ng/mL ούρων για τους μεταβολίτες των PEs, μεταξύ 0.06 και 0.24 ng/mL για τα PBs και 2.01 ng/mL για την BPA. Στην Εικόνα 9 παρατίθεται ένα χαρακτηριστικό χρωματογράφημα πραγματικού δείγματος.

Τα πολύ χαμηλά επίπεδα συγκεντρώσεων της BPA, ιδιαίτερα στα ούρα παιδιών μικρής ηλικίας οδήγησε στην ανάγκη για ακόμα χαμηλότερα όρια ανίχνευσης. Η παραγοντοποίηση της BPA με dansyl chloride μείωσε το mLOD της στα 0.007 ng/mL. Η αντίδραση παραγοντοποίησης ήταν απλή, σύντομη και πραγματοποιούνταν μέσα στο φιαλίδιο (vial) της HPLC μετά την ανάλυση για μεταβολίτες PEs και PBs. Ένα χαρακτηριστικό χρωματογράφημα πραγματικού δείγματος παρατίθεται στην Εικόνα 10. Τυχούσες επιμολύνσεις κατά την επεξεργασία των δειγμάτων δεν ήταν ανιχνεύσιμες. Η μέθοδος εφαρμόστηκε επιτυχώς σε δείγματα προερχόμενα από ένα ανδρικό πληθυσμό ογδόντα (80) ατόμων από την περιοχή της πόλης του Ηρακλείου.

Το πλήρες κείμενο της Εργασίας #1 έχει δημοσιευθεί στο Analytical and Bioanalytical Chemistry (DOI 10.1007/s00216-015-8497-5) και παρατίθεται στη συνέχεια (Appendix 1).

2.2.2. Εργασία #2

Στην παρούσα εργασία παρουσιάζονται τα επίπεδα συγκεντρώσεων επτά μεταβολιτών των PEs, έξι PBs και της BPA σε δείγματα από διακόσια τριάντα εννέα ζευγάρια μητέρας (4ος μήνας εγκυμοσύνης) - παιδιού (2.5 έτη), προερχόμενα από τη μελέτη «ΡΕΑ». Τα επίπεδα συγκεντρώσεων και στους δυο πληθυσμούς ήταν σε συγκρίσιμα επίπεδα με αντίστοιχες εργασίες από άλλες χώρες. Σε τρεις PEs (DEP, DnBP και BBP) και στο EPB βρέθηκαν ασθενείς αλλά στατιστικά σημαντικές συσχετίσεις (CC 0.1-0.2, p<0.01) μεταξύ των επιπέδων στις μητέρες κατά την εγκυμοσύνη και στα παιδιά περίπου τρία χρόνια αργότερα (Πίνακας 1). Συνυπολογίζοντας το χρόνο ημιζωής αυτών των ενώσεων στον ανθρώπινο οργανισμό, με διάρκεια λίγων ημερών διαφαίνεται η ύπαρξη κοινών και σταθερών πηγών εκπομπής στη διάρκεια του χρόνου. Επίσης η ηλικία των παιδιών συσχετίστηκε αρνητικά με τα επίπεδα συγκεντρώσεων όλων των μελετώμενων ενώσεων.

Υπολογίστηκε η ημερήσια πρόσληψη ανά kg σωματικού βάρους και συγκρίνοντας τις διάμεσες τιμές, τα παιδιά βρέθηκαν να έχουν είχαν χαμηλότερα επίπεδα πρόσληψης σε σχέση με τις εγκυμονούσες ημέρες σε όλες τις μελετώμενες ενώσεις εκτός από το DEHP και την BPA. Η ανάλυση κυρίων συνιστωσών (Principal component analysis, PCA) ομαδοποίησε την έκθεση σε δυο κύριες κατηγορίες ανά ομάδα πληθυσμού (Πίνακας 2). Η έκθεση φαίνεται να γίνεται σε μίγματα των ενώσεων και πιο συγκεκριμένα σε μίγματα PEs και BPA (παράγοντας 1 - παιδιά, παρ. 2 - εγκυμονούσες, Πίνακας 2), όπου χρησιμοποιούνται κατά κόρον στο πλαστικό και σε μίγματα PBs και DEP (παρ. 3 - παιδιά, παρ. 4 - εγκυμονούσες), που χρησιμοποιούνται ευρύτατα στα προϊόντα προσωπικής φροντίδας-υγιεινής.

Τέλος, μελετήθηκε ο μεταβολισμός του DEHP, υπολογίζοντας τους σχετικούς μεταβολικούς ρυθμούς (ταχύτητα μετατροπής των μεταβολιτών σε δευτερεύοντες μεταβολίτες) και βρέθηκαν διαφοροποιήσεις στις ταχύτητες μετατροπής ανάμεσα στις εγκυμονούσες και τα παιδιά όπως και ανάμεσα στα αρσενικά και θηλυκά παιδιά.

Το πλήρες κείμενο έχει κατατεθεί για δημοσίευση στο Environment International στις 11/01/2015 και παρατίθεται στη συνέχεια (Appendix 2).

2.2.3. Εργασία #3

Σε συνέχεια της Εργασίας #2, μετρήθηκαν τα επίπεδα των συγκεντρώσεων των μεταβολιτών των PEs, BPA και PBs σε ένα πληθυσμό πεντακοσίων (500) παιδιών προσχολικής ηλικίας (4 ετών), που συμμετέχουν στη μελέτη «ΡΕΑ». Τα επίπεδα συγκεντρώσεων και στα 4 έτη είναι συγκρίσιμα με αντίστοιχες μελέτες εκτός από τα PBs, τα οποία αν και μειωμένα σε σχέση με τα παιδιά 2.5 ετών βρίσκονται σε σημαντικά υψηλότερα επίπεδα σε σχέση με μια αντίστοιχη εργασία που έγινε στη Δανία. Ωστόσο, δεν μπορούν να εξαχθούν ασφαλή συμπεράσματα αν δεν γίνουν περισσότερες σχετικές μελέτες. Η υπολογισμένη ημερήσια πρόσληψη ανά kg σωματικού βάρους ήταν χαμηλότερη στα παιδιά προσχολικής ηλικίας συγκριτικά με τα παιδιά 2.5 ετών και τις εγκυμονούσες γυναίκες (Εικόνα 11). Σε ορισμένες περιπτώσεις ωστόσο, υπερέβη τα θεσμισμένα ανώτατα όρια της Αμερικάνικης Υπηρεσίας Προστασίας Περιβάλλοντος (U.S. Environmental Protection Agency Tolerable Daily Intake) όπως και της Ευρωπαϊκής Αρχής Ασφάλειας Τροφίμων (European

Food Safety Authority Reference Doses). Υπολογίστηκαν επίσης, με βάση περιβαλλοντικές (αέρας εσωτερικού χώρου και πόσιμο νερό) μετρήσεις επιπέδων PEs στην περιοχή του Ηρακλείου, η ημερίσια πρόσληψη μέσω της αναπνοής και της κατανάλωσης πόσιμου νερού. Η συνεισφορά και των δυο αυτών μονοπατιών έκθεσης είναι χαμηλή σε σχέση με την ολική ημερίσια πρόσληψη που υπολογίστηκε με βάση τις συγκετρώσεις στα ούρα.

Οι πηγές έκθεσης ομαδοποιήθηκαν με αντίστοιχο τρόπο (πλαστικό-είδη προσωπικής υγιεινής και φροντίδας) με τα παιδιά 2.5 ετών και τις εγκυμονούσες γυναίκες. Τέλος, ένα από τα στάδια του μεταβολισμού του DEHP (μετατροπή του mEHP σε mEHHP), το οποίο εκφράζεται από το RMR_1 γίνεται πιο γρήγορα στα παιδιά 4 ετών (Εικόνα 12) και η διαφορά αυτή είναι στατιστικά σημαντική ($p < 0.01$).

Το πλήρες κείμενο έχει κατατεθεί για δημοσίευση στο Chemosphere στις 20/02/2015 και παρατίθεται στη συνέχεια (Appendix 3).

3. Συμπεράσματα

Στην παρούσα μελέτη, αναπτύχθηκε για πρώτη φορά ένα πρωτόκολλο ανάλυσης δειγμάτων ανθρώπινων ούρων με χρήση HPLC-MS/MS για τον ταυτόχρονο προσδιορισμό μεταβολιτών των PEs, PBs και BPA. Επετεύχθη για πρώτη φορά επίσης ο χρωματογραφικός διαχωρισμός των ισο- και κ- ισομερών των PPB και BPB με χρήση συμβατικής HPLC. Συνολικά παρακολουθούνται επτά μεταβολίτες των PEs, έξι PBs και η BPA.

Η μέθοδος αυτή βελτιστοποιήθηκε με γνώμονα την ικανότητα ανάλυσης μεγάλου αριθμού δειγμάτων και εφαρμόστηκε σε συνολικά εννιακόσια ογδόντα οκτώ (988) δείγματα ούρων προερχόμενα από εθελοντές της μελέτης «PEA». Αναλυτικότερα, μετρήθηκαν τα επίπεδα συγκεντρώσεων των παραπάνω ενώσεων σε διακόσιες τριάντα εννέα (239) εγκυμονούσες μητέρες, στα παιδιά τους (239) στην ηλικία των 2.5 ετών και σε ένα πληθυσμό πεντακοσίων (500) παιδιών στην ηλικία των 4 ετών.

Η μελέτη αυτή αποτελεί τη μοναδική τέτοιου τύπου στην Ελλάδα και όσον αφορά στο εύρος των μελετώμενων ενώσεων και τον αριθμό των δειγμάτων αποτελεί σημαντική συνεισφορά στην παγκόσμια βιβλιογραφία. Τα επίπεδα των μετρούμενων ενώσεων συσχετίστηκαν αρνητικά με την ηλικία των παιδιών. Στον πληθυσμό των παιδιών 2.5 ετών, τα αρσενικά βρέθηκαν να έχουν στατιστικά σημαντικά υψηλότερα επίπεδα έξι μεταβολιτών των PEs και του n-PPB. Στον πληθυσμό των παιδιών 4 ετών δεν παρατηρήθηκε διαφοροποίηση των συγκεντρώσεων με βάση το φύλο. Τα επίπεδα των μεταβολιτών των PEs και της BPA στην Ελλάδα δεν διαφοροποιούνται σημαντικά σε σχέση με τις αντίστοιχες διαθέσιμες μελέτες. Τα επίπεδα των PBs βρέθηκαν ιδιαίτερα αυξημένα σε σχέση με μια μελέτη από τη Δανία ωστόσο υπάρχει ανάγκη για περισσότερα δεδομένα ώστε να εξαχθεί κάποιο ασφαλές συμπέρασμα.

Μελετήθηκε ο μεταβολισμός του DEHP, υπολογίζοντας τους σχετικούς μεταβολικούς ρυθμούς (RMR), οι οποίοι εκφράζουν την ταχύτητα μετατροπής του mEHP στο mEHHP (RMR_1) και του mEHHP στο mEOHP (RMR_2). Παρατηρήθηκαν στατιστικά σημαντικές διαφοροποιήσεις μεταξύ των ζευγών μητέρας-παιδιού και αρσενικών-θηλυκών παιδιών.

Η ημερήσια πρόσληψη στα παιδιά 4 ετών ήταν χαμηλότερη συνολικά με τα παιδιά 2.5 ετών σε όλες τις μελετώμενες ενώσεις. Οι εγκυμονούσες γυναίκες είχαν υψηλότερη ημερήσια πρόσληψη σε σχέση με τα παιδιά εκτός από το DEHP και την BPA. Η έκθεση σε PEs μέσω του αέρα και του πόσιμου νερού βρέθηκε να έχει χαμηλή συνεισφορά στη συνολική ημερήσια πρόσληψη.

Κάποιες πηγές έκθεσης φαίνεται να είναι κοινές κατά τη διάρκεια της εγκυμοσύνης και στην ηλικία των 2.5 ετών. Η PCA ομαδοποίησε με τον ίδιο τρόπο τις πηγές έκθεσης και στους τρεις υπό εξέταση πληθυσμούς (εγκυμοσύνη, 2.5 και 4 έτη): I) στο πλαστικό για την BPA και τους PEs και II) στα προϊόντα προσωπικής υγιεινής και φροντίδας για τα PBs και DEP.

Abbreviations list

$^{13}C_6$-EPB: $^{13}C_6$–ethyl paraben

$^{13}C_6$-MPB: $^{13}C_6$–methyl paraben

$^{13}C_6$-nBPB: $^{13}C_6$–n-butyl paraben

$^{13}C_6$-nPPB: $^{13}C_6$–n-propyl paraben

APCI: atmospheric pressure chemical ionization

BBP: butyl-benzyl phthalate

BPA: Bisphenol A

CC: correlation coefficient

C_c: average car concentration

C_h: average home concentration

CID: collision induced dissociation

C_u: metabolite concentration, μg/L

C_w: average concentration in water

DEHP: di-2-ethylhexyl phthalate

DEP: di-ethyl phthalate

DI_a:,daily intake calculated based on air concentration

DiBP: di-iso-butyl phthalate

DI_u: daily intake based on urinary metabolite levels

DI_w:daily intake based on water concentration levels

DnBP: di-n-butyl phthalates

EDs: endocrine disruptors

EHT: elimination half times

EPB: ethyl paraben

E.Coli: *Escherichia Coli*

ESI: electrospray ionization

F$_{ue}$: urinary excretion factor

H.Pomatia: *Helix Pomatia*

HMW: high molecular weight

HPLC: high performance liquid chromatography

iLOD: instrument limit of detection

isoBPB: iso-butyl paraben

isoPPB: iso-propyl paraben

LC: liquid chromatography

LMW: low molecular weight

M: average daily water consumption

mBzP: mono-benzyl phthalate

mEHHP: mono-2-ethyl-5-hydroxy-hexyl phthalate

mEHP: mono-2-ethyl-hexyl phthalate

mEOHP: mono-2-ethyl-5-oxo-hexyl phthalate

mEP: mono-ethyl phthalate

miBP: mono-iso-butyl phthalate

mLOD: method limit of detection

mnBP: mono-n-butyl phthalate

MPB: methyl paraben

MS: mass spectrometer

MW$_1$: molecular weight of phthalate diester

MW$_2$: molecular weight of phthalate metabolite

nBPB: n-butyl-paraben

NC: not calculated

ND: not detected

nPPB: n-propyl paraben

NR: not reported

PBs: parabens

PCA: Principal Component Analysis

PEs: phthalate esters

RfD: reference dose

RMR: relative metabolic rate

RMR$_1$: mEHHP/mEHP molar concentrations ratio

RMR$_2$: mEOHP/mEHHP molar concentrations ratio

RPLC: reversed phase liquid chromatography

S/N: signal to noise

SPE: solid phase extraction

SRM: selected reaction monitoring

TDI: tolerable daily intake

UPLC: ultra-performance liquid chromatography

W: body weight

1. Introduction

1.1 Endocrine disruptors

Endocrine disruptors (EDs) are a group of organic compounds, which cause serious alterations to the normal hormone function in humans and wildlife (World Health Organization, 2012). They interfere with hormone biosynthesis, metabolism or action resulting in a deviation from normal homeostatic control or reproduction in humans (Diamanti-Kandarakis et al., 2009). They disrupt the endocrine system by competing with naturally occurring hormones such as estradiol, or by altering the synthesis and metabolism of these hormones (National Institute of Health, 2010); in addition, there is evidence of reproductive toxicity in laboratory animals and possible health effects in humans (Chapin et al., 2008). Bisphenol-A (BPA), parabens (PBs) and 1,2-diesters of phthalic acids (PEs) are established EDs. Six (6) billion pounds of BPA are produced each year worldwide and over 220,000 pounds of this compound are released yearly into the atmosphere (Burridge, 2003). PEs, with over 18 billion pounds used each year, represent one of the world's high production chemical families (Crinnion, 2010) and PBs, which are used in over 13.200 formulations in nearly all type of cosmetics (Elder, 1984). Human exposure to these chemicals is occurring through the environment, food intake and the use of products containing them, through inhalation, dermal contact and ingestion (ATSDR DEHP, 2001; ATSDR DEP, 1995; ATSDR DnBP, 2001; Meeker, 2010; Soni et al., 2001).

1.2 Phthalate esters

PEs have a variety of common uses. High molecular weight (HMW) PEs are used in plastic as softeners and low molecular weight (LMW) PEs are used in personal care products and pharmaceuticals (Wormuth et al., 2006). Previous animal tests and epidemiological studies have associated exposure to PEs with detrimental effects to reproductive and developmental health, as well as increased risk to cancer (ATSDR DEHP, 2001; ATSDR DEP, 1995; ATSDR DnBP, 2001). PEs normally follow a metabolic pathway in at least two steps, a hydrolysis (phase-I) where the phthalate diester is hydrolysed into the primary metabolite monoester phthalate and is followed (phase-II) by a conjugation in order to form the more hydrophilic glucuronidated metabolite (Calafat et al., 2006).

1.3 Bisphenol-A

The 2,2-bis (4-hydroxyphenyl) propane or bisphenol-A (BPA) is used in industry for the production of many pesticides, resins and polycarbonate plastic. BPA can be found in food and beverage processing, and in many products like dental sealants, personal care products, baby bottles, building materials, flame retardant materials and optical lenses, materials for the protection of window glazing, DVDs, and household electronics (Chapin et al., 2008; Geens et al., 2012; Staples et al., 1998). Human exposure to BPA is linked to heart diseases, diabetes, liver abnormalities, reproduction adverse effects and alterations in the thyroid (Rubin, 2011). BPA is excreted mainly via urine in its free form or in its more hydrophilic glucuronide/sulphate conjugate form (Chapin et al., 2008).

1.4 Parabens

PBs is a group of alkyl esters of p-hydroxybenzoic acid. They have low cost of production and demonstrate high chemical stability, inertness, and low acute toxicity (World Health Organization, 2012). These characteristics made them desirable in industry, as antimicrobial preservatives against mould and

yeast, in cosmetics, in pharmaceuticals and in food and beverage processing (Elder, 1984). PBs occur also naturally in food, wine, and plants (Soni et al., 2005). *In vitro* studies indicate that PB induce the growth of MCF-7 human breast cancer cells and influence the expression of estrogen dependent genes (Byford et al., 2002). In general, PBs are partially hydrolysed by esterases to p-hydroxy-benzoic acid and produce glycine/glucuronide/sulphate conjugates, with increased water solubility that are more amenable to urinary excretion than are the free species (Soni et al., 2005; Wang and James, 2006).

1.5 Aim of the study

In order to assess the exposure of humans, to PEs, PBs and BPA, measurement of the urinary concentration of their metabolites (free species and their conjugates) is essential (Silva et al., 2003; Ye et al., 2006). Several methods suitable for measuring phthalate metabolites, BPA or PBs have been published, but none of them measure all three compound classes (Dewalque et al. 2014, Nicolucci et al. 2013, Silva et al. 2003, Silva et al. 2004). Furthermore, only Ultra Performance Liquid Chromatography (UPLC) has been reported to sufficiently separate the structural isomers of propyl- and butyl- paraben (Perkin Elmer 2011). The elimination half times (EHT) of the above mentioned compounds are low. For example, BPA and DEHP metabolites EHT values are some hours (Koch et al., 2004a; Koch et al., 2005; Volkel et al., 2002). Furthermore, pregnant mothers (their embryos) and children are the most vulnerable populations to endocrine disruptor exposure effects (World Health Organization, 2012). As a consequence, there is need for repeated analyses during pregnancy and early childhood in order to assess exposure levels and possible health outcomes.

We aimed to develop a suitable chromatographic method for assessing human exposure to the above-mentioned important EDs. Our main analytical goals were,

I) to develop a common clean-up procedure for phthalate metabolites, PBs and BPA, present in human urine samples;

II) to separate the structural isomers of propyl-paraben, butyl-paraben and monobutyl phthalate metabolite, by using conventional HPLC, instead of UPLC columns and pumps; and

III) to succeed the lowest possible detection limits for all the above-mentioned EDs. We thus achieved high sensitivity, selectivity and the capability to analyse large numbers of samples in reasonable times, making the method eminently suitable for epidemiological studies. In order to test the applicability and appropriateness of the present method we applied it for the analysis of phthalate metabolites, BPA and PBs in a large number of male urine samples.

The developed method was applied for the determination of seven PEs metabolites, six PBs and BPA (Table 1) in two hundred and thirty nine (239) mother (6th month of pregnancy) - child (2.5-year old) pairs and five hundred (500) 4-year old children in Heraklion, Crete (Rhea cohort). Concerning about ED exposure we aimed:

IV) to evaluate, for the first time, the levels of exposure to PEs, PBs and BPA in Greece in three time points and to compare them systematically with other similar studies worldwide,

V) to investigate the potential correlation in the exposure levels between the mothers and their children,

VI)   to estimate the total daily intake (DI_u) of the PEs, PBs and BPA via urinary metabolite concentrations,

VII)   to evaluate the contribution of drinking water and indoor air to the total phthalate exposure,

VIII)   to assess the patterns of exposure.

Table 1. Studied endocrine disruptors and their method limits of detection (mLOD)

Parent compounds in air and water	Metabolites in urine	Method limit of detection (mLOD)		
		ng/mL urine	ng/m^3 air*	pg/mL water*
di-ethyl phthalate (DEP)	mono-ethyl phthalate (mEP)	0.40	0.02	6.7
di-n-butyl phthalate (DiBP)	mono-n-butyl phthalate (mnBP)	0.25	0.02	9.1
di-iso-butyl phthalate (DnBP)	mono-iso-butyl phthalate (miBP)	0.41	0.02	5.6
di-2-ethylhexyl phthalate (DEHP)	mono-2-ethylhexyl phthalate (mEHP)	0.84	0.05	53.7
	mono-2-ethyl-5-hydroxy-hexyl phthalate (mEHHP)	0.01		
	mono-2-ethyl-5-oxo-hexyl phthalate (mEOHP)	0.18		
butyl-benzyl phthalate (BBP)	mono-benzyl phthalate (mBzP)	0.02	0.13	4.1
methyl paraben (MPB)		0.06	-	-
ethyl paraben (EPB)		0.06	-	-
iso-propyl paraben (isoPPB)		0.13	-	-
n-propyl paraben (nPPB)		0.09	-	-
iso-butyl paraben (isoBPB)		0.04	-	-
n-butyl paraben (nBPB)		0.04	-	-
Bisphenol-A (BPA)		0.01	-	-

*(Katsikantami, 2014; Perraki 2011)

2. Materials and methods

2.1 Analytical standards, reagents and consumables

Mono-ethyl phthalate (mEP), $^{13}C_4$-labeled mEP, mono-n-butyl phthalate (mnBP), $^{13}C_4$-labeled mnBP, mono-iso-butyl phthalate (miBP), mono-benzyl phthalate (mBzP), $^{13}C_4$-labeled mBzP, mono-2-ethyl-hexyl phthalate (mEHP), mono-2-ethyl-5-hydroxy-hexyl phthalate (mEHHP), $^{13}C_4$-labeled mEHHP, mono-2-ethyl-5-oxo-hexyl phthalate (mEOHP), $^{13}C_4$-labeled mNP, 4-methylumbelliferrone, $^{13}C_4$-labeled 4-methylumbelliferrone and D_{16}-Bisphenol-A (D_{16}-BPA) were obtained from Cambridge Isotope Laboratories (USA). 4-methylumbelliferryl glucuronide, $^{13}C_6$-labeled MPB, $^{13}C_6$-labeled EPB, $^{13}C_6$-labeled n-PPB, $^{13}C_6$-labeled n-BPB, dansyl chloride, formic acid (for MS, 98%), solvents (Chromasolv grade for HPLC acetonitrile, ethyl acetate, acetone and methanol) and ammonium hydroxide (28% w/v in water) were purchased from Sigma Aldrich (Germany). Methyl-paraben (MPB), ethyl-paraben (EPB), n-propyl paraben (n-PPB), n-butyl paraben (nBPB), (iso-BPB) iso-butyl-paraben, Bisphenol-A (BPA) and iso-propyl paraben (iso-PPB) were purchased from AccuStandard (USA). Glacial acetic acid was purchased from Carlo Erba (Italy) and orthophosphoric acid (85% w/v in aqueous solution) from Riedel de Haen (Switzerland). Ammonium acetate and monosodium phosphate (reagent grade) were provided by Fluka (Germany). *Escherichia Coli* (*E.Coli*) β-glucuronidase (140 U/mL) was purchased from Roche (Germany). SPE cartridges (Nexus, 60mg sorbent / 3 mL reservoir and 200mg sorbent / 6mL reservoir) were acquired from Varian (USA). High purity water (18.2 MΩ x cm electrical resistivity), was produced by PURELAB Ultra Ionic purification system (ELGA, USA).

2.2 Preparation of standards

All standard solutions were stored sealed at -20 °C in Teflon-capped bottles. Phthalate metabolites and 4-methylumbelliferrone standards (native and labelled) obtained in solutions (100 μg/mL in methyl-tert-butyl ether or acetonitrile). 4-methylumbelliferryl glucuronide standard stock solution was prepared in water at 1000 μg/mL. Dansyl chloride standard solution was prepared in acetone at 12.5 mg/mL. PBs, BPA and D_{16}-BPA stock solutions were prepared in methanol at 250 μg/mL. Working solutions were prepared at concentrations of 1 μg/mL in 1:1 methanol/water for mass spectrometer optimisation and at 2-8 μg/mL in synthetic urine (Laboratory Procedure Manual, Method No: 6301.01., 2009) or spiking samples and calibration curves. Quantitative analysis was based on peak area measurements as ratios with the peak area of their corresponding internal standard. For phthalate metabolites isotopically labelled analogues of native compounds were used as internal standards, except for miBP, mEOHP and mEHP where $^{13}C_4$-mnBP and $^{13}C_4$-mEHHP were used because the labelled analogues were not commercially available to us for the period of the study. For PBs analysis $^{13}C_6$-analogues of methyl-, ethyl, n-propyl and n-butyl parabens were used as internal standards. For BPA analysis, D_{16}-BPA was used as the internal standard. Calibration curve solutions, blank, recovery and quality control (QC) samples were prepared in synthetic urine.

2.3 Instrumentation

All analyses were performed on an LC-MS/MS system consisting of an RP-HPLC chromatograph coupled to a mass spectrometer. Sample injections were performed via a Surveyor Autosampler (Thermo Finnigan, USA). The chromatographic separation of PBs-BPA-phthalate metabolites was achieved using a Surveyor LC system (Thermo Finnigan, USA), equipped with a BetaSil Phenyl (3 μm, 100 mm x 2.1 mm) analytical column from Thermo Scientific (USA). Dansylated-BPA/D_{16}-BPA were analysed with a PerfectSil C_8 (3 μm, 125 mm

x 2.1 mm, MZ-Analytical, Germany) analytical column. The mass detection was achieved with a TSQ Quantum triple quadrupole mass spectrometer equipped with both ESI and APCI source (Thermo Finnigan, San Jose, USA). The system was controlled by the Xcalibur software, which also was used for the data acquisition, analysis and quantitation.

2.4 Mass spectrometry conditions

The mass spectrometer was operated in the selected reaction monitoring (SRM) mode. Source collision induced dissociation (Source CID) and tube lens voltage were set at optimum values for each SRM. Collision gas was Ar at 2.0 mTorr. For PBs-BPA-phthalate metabolites, ESI in negative mode was chosen as the ionization source. Sheath gas pressure (32-45 arbitrary units, au) and auxiliary gas pressure (20 au) were N_2 and as with spray voltage (4000-4400 V) and Lens 0 offset (1.0-1.3 V), optimum values were set at each time segment. Ion transfer capillary temperature was set at 330°C.

Table 2. Selected reaction monitored for phthalate metabolites, parabens, BPA, dansylated-BPA and their isotopically labelled analogues

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
MPB	151.1	92.1	14
$^{13}C_6$-MPB	157.1	98.1	14
EPB	165.1	92.1	30
$^{13}C_6$-EPB	171.1	98.1	30
nPPB / isoPPB	179.1	92.1	40
$^{13}C_6$-nPPB	185.1	98.1	40
nBPB / isoBPB	193.1	92.1	33
$^{13}C_6$-nBPB	199.1	98.1	30
mEP	193.1	77.1	10
$^{13}C_4$-mEP	197.1	79.1	10
mnBP / miBP	221.1	77.1	10
$^{13}C_4$-mnBP	225.1	79.1	10
mEHHP	293.2	121.1	10
$^{13}C_4$-mEHHP	297.2	124.1	10
mEOHP	291.2	121.1	10
mBzP	255.2	105.1	10
$^{13}C_4$-mBzP	259.2	107.1	10
mEHP	277.2	134.1	10
BPA	227.2	212.2	18
D_{16}-BPA	241.2	223.2	19
4methyl-umbelliferrone	177.1	133.1	26
$^{13}C_4$-4methyl-umbelliferrone	179.1	135.1	26
Dansylated BPA quantitation SRM	695.5	171.1	40
Dansylated BPA confirmation SRM	695.5	235.1	38
Dansylated D16-BPA	709.5	170.1	35

The monitored SRMs of the studied compounds and their isotopically labelled internal standards are presented in Table 2. Dwell time was set at 0.1-0.2 sec except for BPA in which case it was set at 1.5 sec. Dansylated-BPA was measured with ESI in the positive mode. Sheath gas pressure (35 au) and auxiliary gas pressure (20 au) were N_2. Spray voltage was set at 4000 V and Lens 0 offset at 0.4 V. Ion transfer capillary temperature was set at 300°C. The SRMs are depicted in Table 2. Dwell time was set at 0.75 sec for quantitation ion, 0.55 sec for confirmation ion and 0.2 sec for D_{16}-BPA ion.

2.5 HPLC conditions

Injection volume was 20 µL and autosampler settings were as follows: flush volume 1600 µL, wash volume 1600 µL, flush speed 100 µL/s and as wash/flush solvent was used methanol-water 1:1. For PBs-BPA-phthalate metabolites, we modified the gradient used by (Silva et al., 2004) as depicted in Table 3. Flow rate was set at 350 µL/min. For dansylated-BPA analysis, the applied gradient (200 µL/min flow rate and 0.1% formic acid as mobile phase additive) is presented in Table 4.

Table 3. Gradient program for phthalate metabolites paraben and BPA analysis

Time (min)	(0.1 % acetic acid in acetonitrile) %	(0.1 % acetic acid in water) %
0	4	96
0.1	4	96
1.0	15	85
14.0	25	75
27.0	35	65
28.0	100	0
32.0	100	0
33.0	4	96
36.0	4	96

Table 4. Gradient program for dansylated-BPA analysis

Time (min)	(0.1 % formic acid in acetonitrile) %	(0.1 % formic acid in water) %
0	60	40
0.1	60	40
15.5	100	0
17.0	100	0
17.1	60	40
18.4	60	40

2.6 Sample preparation

After collection, samples were stored at -18 °C and were thawed overnight at 4 °C before analysis. Treatment and clean-up of the samples was based on previous work (Silva et al., 2003b) but modified as follows: Urine samples (1 mL) were transferred to a Falcon tube (polypropylene, 15 mL) and spiked with 100 ng $^{13}C_4$-labeled phthalate metabolites and $^{13}C_4$-labeled 4-methylumbelliferrone, 20 ng $^{13}C_6$-labeled PBs and 200 ng 4-methylumbelliferryl glucuronide. The hydrolysis step, with use of *E.Coli* or *H.Pomatia* β-glucuronidase, has been reported and evaluated in numerous publications (Chen et al., 2012; Dewalque et al., 2014; Ye et al., 2005). We have used the *E.Coli* β-glucuronidase hydrolysis as follows: *E.Coli* β-glucuronidase buffer (prepared daily, per sample: 10 µL *E.Coli* β-glucuronidase and 250 µL ammonium acetate buffer, 1M in aqueous solution, pH 6.5) was added to the urine samples and hydrolysis was completed at 37 °C for 90 min. After enzymatic hydrolysis completion, 1 mL of ammonium hydroxide buffer (0.15 % w/v

NH₄OH in 1:1 acetonitrile-water) was added to the samples, which were loaded onto the 60 mg solid phase extraction cartridge. The eluents of the first cartridge (60 mg) were acidified with 3 mL monosodium phosphate buffer (0.14 M NaH_2PO_4, aqueous solution, at pH 2) and loaded onto the second solid phase extraction cartridge (200 mg). The eluents from the 200 mg cartridge were discarded. Both cartridges were eluted with 3 mL acetonitrile and 3 mL ethyl acetate each. The eluents of both cartridges (12 mL in total) were combined and evaporated to dryness with a rotational vacuum concentrator RVC 2-25 (Martin Christ, Germany) (60 °C, 20-45 mbar, 150 min for 18 samples). The residues were dissolved in 0.4 mL of water and transferred to a 2 mL autosampler glass vial with a 0.4 mL volume insert. After phthalate metabolites-PBs-BPA LC-MS analysis, in order to enhance BPA detection limit, 160 μL 7% v/v aquatic ammonium hydroxide and 40 μL dansyl chloride 12.5 mg/mL in acetone were added to the autosampler vials containing the samples (200 μL, the rest was discarded) and with 0.5h heating at 65 °C, dansylation was completed and samples were re-analysed with LC-MS. In order to normalise the variability in urine density, an aliquot of 0.5 mL for each urine sample was analysed to determine the creatinine by concentration using the OLYMPUS 2700 immunoassay system (Beckman Coulter, USA).

2.7 Analytical performance

The following parameters were evaluated for the analytical performance of the method: isotopic purity of labelled compounds, recovery and blank levels, method limit of detection/quantitation (mLOD/mLOQ), method limit of detection/quantitation (mLOD/mLOQ), linearity, accuracy and repeatability. Matrix effects cannot be calculated accurately due to the variability of urine density among samples. For this reason, an average matrix effect influence was taken into account for the analyses, by using synthetic urine in calibration curves, blanks, recovery and quality control samples. In order to check the isotopic purity of labelled compounds, we analysed an aqueous solution (200 ng/mL) of each standard, three times. To determine the method recovery, 1 mL of synthetic urine (spiked with native analytes: 1, 5 ng and 50 ng for PBs / BPA and 5, 50 and 100 ng for phthalate metabolites) was analysed three times for each level and internal standards were added before HPLC-MS analysis. To determine possible contaminations during analysis (blank levels), 1 mL of synthetic urine was analysed three times. Instrument limits of detection (iLOD) and quantitation (iLOQ) were set at signal to noise (S/N) ratios equal to 3 and 10 respectively. iLOD and iLOQ were calculated using a calibration curve in synthetic urine in order to take into account signal suppression due to matrix effect. The mLOD and mLOQ were calculated by adjusting iLOD and iLOQ respectively, with the method recovery value and sample condensation factor using the following equations: [mLOD] = [iLOD] / [sample condensation factor * recovery] & [mLOQ] = [iLOQ] / [sample condensation factor * recovery]. The linearity of the method (R^2) was calculated by using the linear equation of calibration curve for each analyte. In order to evaluate the repeatability of the method, a pooled urine sample, spiked with native analytes (1, 5 and 50ng for PBs/ BPA and 5, 50 and 100 ng for phthalate metabolites) was aliquoted and analysed five times for each level. The accuracy of the method was calculated by analysing quality control QC samples (N=5 for each level), which were prepared by spiking the same amounts of native compounds as in repeatability test to synthetic urine.

2.8 Study population

The present study is part of the "Rhea" project, a pregnancy cohort which examines prospectively a population-based cohort of pregnant women and their children at the prefecture of Heraklion, Crete, Greece (Chatzi et al., 2009; Patelarou et al., 2011). Briefly, women who became pregnant during February 2007-

February 2008 participated in the study. Women, residents of the study area, >16 years of age, completed face-to-face interviews and provided blood and urine samples, visiting a participating hospital or private clinic during the 10th–13th week of gestation. The next contacts with the mothers were at 24 weeks of gestation, at birth, at 8-10 weeks after delivery and for child's follow-up at 9th, 18th months, and at 4 years of age.

Children (2.5 year old) samples (103 females-136 males) were collected during March 2009-June 2011. Spot urine samples were collected at around the fourth month of pregnancy for mothers and at 2.3 ± 0.72 years for children; mean age ± standard deviation). A total of 239 mother-child pairs were monitored. Of 1363 singleton live births in the Rhea study, 879 children participated at the 4 years follow up, during which urine samples were obtained from 800 children. Of them, a random subset of 500 children (221 females-279 males, 4.24 ± 0.24 years old; mean age ± standard deviation) was included in the present analysis. The study was approved by the Ethical Committee of the University Hospital of Heraklion (Crete, Greece) and all participants provided written informed consent.

Urine samples were collected in urine boxes and stored at 4°C until procession. Within 4 hours, samples were aliquoted in 4mL cryovials and stored at -80°C. Urine boxes and cryovials were made of polypropylene and checked for possible contaminations. Creatinine levels were 0.50±0.31 g/L (arithmetic mean ± standard deviation) for 2.5 years old children 0.70±0.36 g/L for 4 years old children and 1.20±0.67 for mothers. Samples with creatinine values, out of 0.3-3 g/L range for mothers (Barr et al., 2005a; Barr et al., 2005b) and out of 0.1-3.0 range for children were excluded from analysis. We did not applied the same exclusion criteria for mothers and children, because creatinine values below 0.3 mg/L in children do not necessarily indicate excessive dilution but are indicative of lower muscle mass compared to adults (Koch et al., 2011). All participant mothers provided written, informed consent for themselves and their child after having received a complete description of the study, which was approved by the Ethics Committee of the University Hospital in Heraklion, Greece.

2.9 Environmental samples

Environmental phthalate analyses results have been provided by Environmental Chemical Processes Laboratory (Katsikantami, 2014; Perraki 2011). Briefly, ten houses were selected in Heraklion area (study area of the Rhea cohort) for monitoring phthalate esters indoor air levels. Moreover the air concentrations of PEs were determined in the interior of ten used private cars of Heraklion citizens. Indoor air sampling was conducted with a Buck sampling pump (flow rate: 5L/min; duration: 9-18h; Sigma Aldrich, USA) equipped with polyurethane foam filters (diameter: 5.5 cm; height: 8 cm) during April-July 2011. Moreover forty-nine tap water samples were collected from November 2013 up to September 2014 also in the study area of the Rhea cohort (Goslan et al., 2014). Water samples were collected in 250-ml glass bottles with glass caps, during the morning in order to monitor PEs levels in water, which had remained several hours inside the in-home water network. Sampling was performed from November 2013 up to September 2014.

Five phthalate diesters (parent compounds of the urinary phthalate metabolites) were determined in tap water and indoor air. Their mLODs are reported in Table 1. The procedure for the water analysis was based on a previously described protocol (Environmental Protection Agency, 1995) slightly modified as follows: 100 mL of water were spiked with surrogate standard (deuterated-DEHP) and liquid-liquid extracted three times

with 5 mL dichloromethane (15 mL total). Extracts were then loaded onto a Pasteur pipette filled with Na_2SO_4 (5g, anhydrous) in order to remove water residues. At a next step dried extracts were concentrated to 100 µl with a vacuum rotational evaporator, followed by a gentle N_2 stream. The samples were spiked with internal standard (benzyl benzoate) and finally were analysed with GC-MS.

Linearity was excellent (R^2>0.99, 0.6-50 µg/mL), recoveries (n=5) ranged from 63 to 110%; repeatability tests at two different levels (800 and 10000 ng/L) showed standard deviation <3.44%. Average blank contamination was: DEP 1.6 ng; DiBP 3.9 ng; DnBP 2.1 ng; DEHP 22.9 ng; BBP <LOD, (standard deviation < 11.5%). In every four water samples, a blank sample was also analysed. For the analysis of indoor air samples, polyurethane foams were spiked with surrogate standard (deuterated-DEHP) and processed using accelerated solvent extraction (oven temperature 90°C; pressure: 1500psi; heating time: 5min; static time: 5min; number of cycles: 1; flush volume: 60%; purge time: 1min). The extracts were condensed to 500 µL with a vacuum rotational evaporator. Then with a gentle N_2 stream were condensed to 100 µL, were spiked with internal standard (benzyl benzoate) and finally were analysed with GC-MS. Linearity was excellent (R^2>0.99, 0.6-50 µg/mL) and recoveries (n=5) ranged from 73.3% to 90.3% (standard deviation < 12.3%). Blank contamination was: DEP 9.6 ng; DiBP 33.8 ng; DnBP 22.2 ng; DEHP 82.4 ng; BBP 14.0, <82.4 ng (standard deviation <33% except BBP with 107%). In every five air samples, a blank sample was also analysed. The GC-MS system was consisted of an Agilent GC 6890N/MSD 5973 equipped with an Autosampler HP 7683. The GC-MS parameters were as follows: 1 µL on-column injection; capillary column DB-5MS (30 m, 0.25 mm i.d., 0.25 µm film thickness); carrier gas helium; constant velocity 33 cm/s; transfer line 290°C; temperature program: initial temperature 60°C, 20°C/min to 180°C, 10°C/min to 290°C, held for 10 min, total duration 27 min; electron impact at 70 eV; selected ion monitoring mode.

2.10 Statistical analysis

Statistical analysis was performed with the software SPSS 22.0 (IBM Corporation, U.S.A.). Measurements below mLOD (not detected) were substituted by the mLOD divided by the square root of 2 (two) (Hornung 1990), as the most widely used way to handle non-detects in such type of studies (Ferguson et al., 2014; Song et al., 2013). Arithmetic mean, minimum, median, 95th percentile, maximum, geometric mean and 95% confidence interval of geometric mean (95 % CI) values were calculated for both unadjusted/creatinine-adjusted concentrations and estimated total daily intake data.

The daily intake of the studied EDs was estimated by adapting a commonly used toxicokinetic model to our data (Equation 1) (Beko et al., 2013; Dirtu et al., 2013; Ma et al., 2013), where: DI_u (Daily Intake calculated using urinary metabolites, µg×d^{-1}×kg^{-1} of body weight), C_u (metabolite concentration, µg/L), F_{ue} (urinary excretion factor, molar ratio of parent compound in taken to metabolite excreted), MW_1 (molecular weight of PE, g/mol), MW_2 (molecular weight of PE metabolite, g/mol) and W (body weight, kg). Especially for PBs and BPA ratio (MW_1/MW_2) was set equal to 1.

F_{ue} values for mEHHP and mEOHP were taken from (Koch et al., 2004a) and (Koch et al., 2005a), for mBzP and mnBP from (Anderson et al., 2001), for PBs from (Ma et al., 2013) and since for miBP and mEP, F_{ue} values were not available, we used the same with mnBP. F_{ue} value for BPA was set equal to 1 since BPA is excreted via urine nearly 100% during an 24h period (Volkel et al., 2002). V_u considered 2 L for mothers

(Guo et al., 2011) and 0.0224 L/kg body weight for children (Miller and Stapleton 1989; Szabo and Fegyverneki 1995). We chose to use a value for children volume urine, which doesn't take into account body weight when it is applied to our toxicokinetic model because children especially in ages of this study grow rapidly and a stable volume of urine as in used mothers could introduce uncertainty in daily intake estimation.

Furthermore, the daily intakes from air (DI_a) and water (DI_w) were estimated for 4 years old children using Equations 2 and 3 respectively. Since air and water concentration data were not available for each cohort subject, we used the average air and water levels in Heraklion area for our calculations. Especially, for DI_a (Equation 2), we supposed that a children (average body weight of children participating in this study, W:18.4 kg) spends 22.5 h (95.5%) daily indoor and ≈1.5 h (5.5%) in car (C_h: average home concentration; C_c: average car concentration) and inhales 8.3 m^3/d (Wilson et al., 2001); for DI_w, the average concentration in water (C_w), the average body weight as in Equation 2 and an average daily water consumption (M: 1L/d) (World Health Organisation, 2008).

In order to investigate possible differentiations in DEHP metabolism among population groups, relative metabolic rate (RMR) of DEHP was calculated as described in literature (Boas et al., 2010; Song et al., 2013). Briefly, RMR_1 (1st step of metabolism) considered as the molar concentration ratio of mEHP/mEHHP and RMR_2 (2nd step) the ratio of mEHHP/mEOHP. Only samples with positive detection in all three DEHP metabolites were used. For two-tailed Spearman correlations, creatinine adjusted molar (μmol/g) concentrations were used. For two-tailed Pearson correlations, unnormalised RMR values were used since RMR data were not skewed.

Principal component analysis (PCA) with Kaiser normalisation, Mann-Whitney U (concentration levels gender-based comparison) and Wilcoxon signed ranked (comparison of the same children at 2.5 and 4 years and mother-child pairs daily intake) tests were applied to unadjusted for creatinine, log10 transformed concentrations. Independent samples t-test was applied to unnormalised RMR data (mothers-children as independent populations and male-female children comparisons) and to gender-based creatinine levels comparison. For PCA and correlation studies, molar concentration levels were used and mEHHP-mEOHP concentrations summed as DEHP metabolites.

Analytes with detectability lower than 50.0% (isoPPB, isoBPB and nBPB) were excluded from geometric mean calculation and correlation studies. For PCA analysis, isoPPB and isoBPB were excluded for the same reasons while nBPB was included (e.g. detectability: 37.6% at 4-years old children). DEHP-DI_u considered as the arithmetic mean of DI_u for mEHHP and mEOHP. mEHP was excluded from the above analyses (PCA, correlation studies, DI_u) due to its relatively lower levels in urine and shorter half-life compared to the other two measured DEHP metabolites, mEHHP and mEOHP (Frederiksen et al., 2007; Koch et al., 2005; Silva et al., 2006a; Silva et al., 2006b; Wittassek and Angerer, 2008).

Equation 1: $DI_u = \frac{C_u \times V_u \times MW_1}{W \times F_{ue} \times MW_2}$ (μg of ED × d^{-1} × kg^{-1} of body weight)

Equation 2: $DI_a = \frac{[(C_h \times 0.945) + (C_c \times 0.055)] \times 8.3}{W}$ (μg of ED × d^{-1} × kg^{-1} of body weight)

Equation 3: $DI_w = \frac{C_w \times M}{W}$ (μg of ED × d^{-1} × kg^{-1} of body weight)

2.11 Comparison with other studies worldwide

Literature search for similar studies was performed via EndNote X7 (Thompson Reuters) in PubMed database on December 04, 2014. The search criteria were the following: for PEs metabolites/BPA, titles containing (*phthalate or bisphenol-a or bpa) and (child* or mother* or pregnan* or women) and for PBs titles containing *paraben*. The selection criteria were: for studies measuring total metabolites (free, glucuronated and sulphated) from pregnant women or children for over 200 urine samples for PEs metabolites/BPA and 100 for PBs, with creatinine normalised median concentrations available for BPA or at least for 4 common PEs metabolites or 4 common PBs with our study.

Initially, 796 articles were identified: 93 hits for (*phthalate* and child*), 10 for (*phthalate* and mother*), 63 for (*phthalate* and pregnan*), 46 for (*phthalate* and women), 44 for (bisphenol-a and pregnan*), 53 for (bisphenol-a and child*), 8 for (bisphenol-a and mother*), 41 for (bisphenol-a and women), 3 for (bpa and women), 1 for (bpa and mother*), 2 for (bpa and pregnan*), 4 for (bpa and child*) and 428 for *paraben*. Fifteen (15) of them fulfilled the search criteria: (Boas et al., 2010; Braun et al., 2011; Braun et al., 2009; Casas et al., 2013; Frederiksen et al., 2013; Harley et al., 2013; Hong et al., 2013; Kasper-Sonnenberg et al., 2014; Lee et al., 2014; Mortamais et al., 2012; Quiros-Alcala et al., 2013; Tefre de Renzy-Martin et al., 2014; Teitelbaum et al., 2012; Wang et al., 2014; Zeman et al., 2013). References of the selected papers were also checked but no additional articles identified. In case of concentrations given separately for male/female children or at different time-points of pregnancy, the arithmetic mean of the given median values was used.

3. Results and discussion

3.1 Optimization of mass spectrometry

ESI is the widely used ionisation technique due to its enhanced sensitivity, the low flow rates it requires and its capability to ionise a wide range of analytes (Chen et al., 2012; Laboratory Procedure Manual, Method No:6301.01., 2009; Dewalque et al., 2014; Silva et al., 2004). Although, sensitivity was at same levels with APCI, the need for frequent maintenance of this ionisation source (cleaning/replacing corona needles, replacing sample tube) and the significantly larger solvent consumption led us to use ESI. A preliminary optimization of the mass spectrometer parameters took place with direct infusion of each compound at 10 µL/min flow rate via a syringe pump. Optimization was repeated, after HPLC method development, and each compound was re-optimized using the chromatographic conditions (flow rate and solvent type), existing at its retention time. Mobile phase flow (via HPLC pump) and analyte solution flow (via syringe pump) were connected with a T-junction and were driven to the mass spectrometer. Despite the automatic optimization capability of TSQ Quantum, in order to achieve more accurate results, we performed this step manually. In order to achieve appropriate detection limits for phthalate metabolites, PBs and BPA with our mass spectrometer (TSQ Quantum, model acquired in 2003), we chose to follow one SRM per analyte. Furthermore, we have tested and optimized two SRMs per analyte without observing co-eluting peaks in any sample. For dansylated-BPA, two SRMs were monitored.

3.2 Optimisation of HPLC

For PBs-BPA-phthalate metabolites and with ESI as ionisation source, in addition to the selected BetaSil Phenyl column, we also tested a PerfectSil 120 Phenyl (3 µm, 100 mm x 2.1 mm; MZ-Analytical, Germany) and a Gemini C_{18} (3 µm, 100 mm × 2 mm; Phenomenex, USA) HPLC columns, which did not provide adequate separation and peak shapes. Methanol was tested as the mobile phase; although for PBs and BPA analysis the results were similar to those obtained using ACN, for phthalate metabolites the separation and peak shapes were unsatisfactory. With APCI, a pair of tandemly connected Hypersil ODS (5 µm, 250 mm × 4.6 mm, MZ-Analytical, Germany) HPLC columns were tested, with both ACN and methanol used as mobile phases. Although separation for PBs was similar to that achieved using a BetaSil Phenyl column with ESI, the gradient was longer, the system pressure and flow rate were significantly higher and the separation of phthalate metabolites was inadequate. The optimum results for the baseline separation of the structural isomers of BPB, PPB and mBP, were accomplished with the BetaSil Phenyl HPLC column. The modified gradient (of a previously reported method (Silva et al., 2004); see "Material and methods - HPLC conditions") we used (Table 3), completed the separation of paraben stuctural isomers. The addition of 0.1% acetic acid to the mobile phase was essential for the retention of phthalate metabolites and their proper separation (Chen et al., 2012; Dewalque et al., 2014; Silva et al., 2004). Besides, the acidic mobile phase suppresses the analyte signal, in the negative ESI mode and increases the iLOD for all studied analytes (Table 5) and particularly for BPA (Chen et al., 2012; Dewalque et al., 2014; Silva et al., 2004). A chromatogram of a pooled urine sample, spiked with 100 ng of all analytes, is shown in Figure 1.

For dansylated-BPA analysis (structure is presented in Figure 1), between PerfectSil C_8, BetaSil Phenyl and Gemini C_{18} columns, the first demonstrated the best chromatographic performance (peak shape, S/N,

matrix components separation). The new gradient, we developed, is presented in Table 4, and the chromatographic result of its use for the analysis of a real urine sample is depicted in Figure 3.

Table 5. Comparison of instrument / method Limits of Detection / Quantification for different conditions

Analyte	iLOD-iLOQ (ng/mL)			mLOD-mLOQ (ng/mL)		
	with 0.1% acetic acid (ng/mL)	without acetic acid (ng/mL)	with dansylation (ng/mL)	with acetic acid in mobile phase	without acetic acid in mobile phase	with dansylation
mEP	-	-	-	0.40-1.33	-	-
mnBP	-	-	-	0.25-0.83	-	-
miBP	-	-	-	0.41-1.37	-	-
mBzP	-	-	-	0.02-0.07	-	-
mEHP	-	-	-	0.84-2.80	-	-
mEHHP	-	-	-	0.01-0.03	-	-
mEOHP	-	-	-	0.18-0.60	-	-
MPB	0.28-0.93	0.14-0.47	-	0.12-0.40	0.06-0.20	-
EPB	0.13-0.43	0.12-0.40	-	0.06-0.20	0.06-0.18	-
iso-PPB	0.41-1.37	0.23-0.77	-	0.24-0.80	0.13-0.45	-
nPPB	0.33 -1.10	0.19-0.63	-	0.15-0.50	0.09-0.29	-
iso-BPB	0.15-0.50	0.07-0.23	-	0.08-0.27	0.04-0.13	-
nBPB	0.15-0.50	0.08-0.27	-	0.07-0.23	0.04-0.12	-
BPA	4.43-14.76	0.16-0.53	0.008-0.026	2.01-6.69	0.07-0.24	0.007-0.024

In order to increase reproducibility and zero carry-over effects in HPLC, a mixture of 1:1 methanol-water was chosen as syringe cleaning solvent and syringe washes were modified as described in the "Material and methods - HPLC conditions" section. To the best of our knowledge, there is only one report using a UPLC column for the separation of both paraben structural isomers (butyl- and propyl-) [30]. Another study [39], applied an unpublished method, which separates both propyl- and butyl- although only few details are given. In the present study, we achieved for first time, to separate these three isomers using a conventional HPLC column and pump.
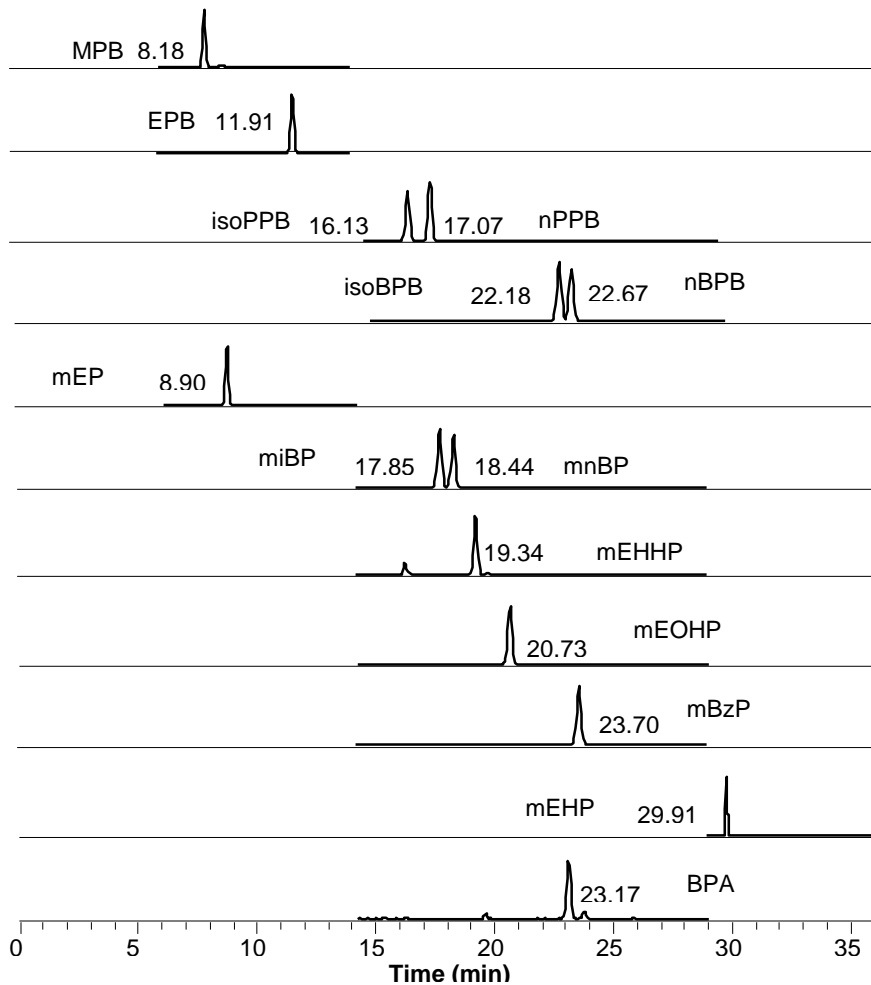
Figure 1. Pooled urine sample chromatogram for simultaneous PEs metabolites / PBs / BPA analysis and their SRMs, peak intensities and retention times in minutes
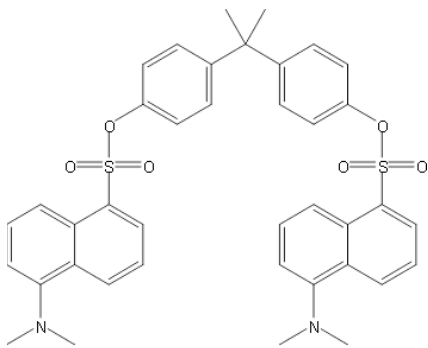
Figure 2. Dansylated BPA structure
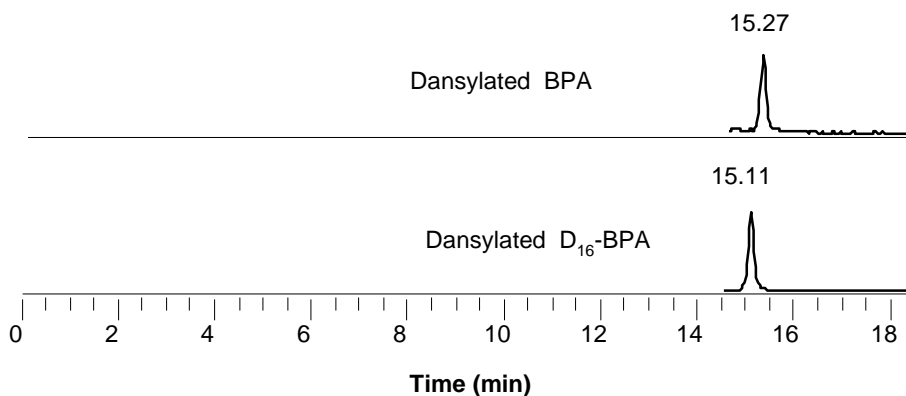
Figure.3 Urine sample chromatogram for dansylated BPA / D$_{16}$-BPA and their SRMs, peak intensities and retention times in minutes

3.3 Optimisation of sample preparation

Applying the modified clean-up procedure for phthalate metabolites (see "Materials and methods-sample separation"), PBs and BPA were also effectively retained (Table 6). Furthermore, to analyse more samples simultaneously and minimize manual intervention, evaporation to dryness was performed with the rotational vacuum concentrator. Derivatisation of phenolic hydroxyl groups with dansyl chloride is a well-known reaction in organic chemistry and it has applied to enhance mass spectrometric sensitivity of urinary BPA (Fox et al., 2011, Wang et al., 2013). We used aqueous NH$_4$OH to buffer the samples, instead of a non-volatile buffer (Fox et al., 2011, Wang et al., 2013) in order to prevent blocking of ion transfer tube after a few injections. Basic pH does not affect BPA deprotonation at ESI source and therefore does not suppress its signal since it elutes at 15.1 min (NH$_4$OH is not retained by the column) and the eluent contains 0.1% formic acid. Dansyl chloride added in excess (0.5 mg per sample) to ensure dansylation for any urine sample (variable concentrations of compounds with phenolic hydroxyls). To the best of our knowledge, this is the first common clean-up protocol reported for the analysis of phthalate metabolites, PBs and BPA

3.4 Analytical performance

Recoveries were higher than 59.1% for all studied metabolites and spiking levels except mEHP (41.5 - 43.5%), which was not eluted effectively from the SPE cartridges possibly due to its high lipophilicity (Table 6). We consequently achieved method limits of detection at the pg/mL - low ng/mL range. Blank contamination was not detectable. The linearity, for the expected concentration range (as presented in Table 6), was excellent ($R^2 > 0.99$). All isotopically labelled standards were found without detectable contaminations of native compounds. Matrix effects have been reported, for both APCI and ESI analyses, of the studied compounds (Chen et al., 2012; Dewalque et al., 2014; Silva et al., 2003). In order to control matrix effects and to perform an accurate analysis, we have used isotopically labelled internal standards for most of the target compounds. Due to their limited commercial availability in the period of study, we used nine labelled internal standards for fourteen target compounds. Repeatability experiments showed standard deviations (STD) <15.0% and accuracy <20.8% for all studied metabolites and spiking levels (Table 6). The chromatogram of a spiked

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pooled urine sample, analysed for phthalate metabolites-PBs-BPA, is shown in Figure 2 and a urine sample, processed with dansyl chloride, is presented in Figure 3.

Table 6. Analytical performance characteristics

Compound	Linearity range (R^2>0.99) (ng/mL)	Recovery % (n=3, ± SD)			Accuracy %			Repeatability %		
		Low	Medium	High	Low	Medium	High	Low	Medium	High
mEP	0.5-512	60.5±6.4	69.5±4.5	68.5±7.5	9.2	5.5	7.3	2.6	2.6	5.2
mnBP	0.5-512	80.7±7.8	87.2±7.8	70.6±8.2	5.7	6.6	4.2	2.0	7.0	3.5
miBP	0.5-512	73.9±9.6	76.7±5.4	67.3±7.4	9.0	3.9	3.1	6.2	2.7	4.2
mBzP	0.5-512	64.8±5.4	69.4±6.6	67.0±6.7	7.4	4.0	3.8	6.8	5.5	3.5
mEHP	1-512	43.5±5.2	40.8±1.8	41.5±16.0	20.8	19.3	5.2	7.9	2.8	5.7
mEHHP	0.5-512	64.2±7.8	62.1±1.8	66.6±7.5	9.1	7.6	6.3	5.2	5.7	5.6
mEOHP	0.5-512	59.1±5.1	79.6±7.9	68.3±7.1	15.6	8.5	7.0	6.9	7.6	6.2
MPB	0.25-512	95.0±8.6	99.8±4.3	91±3.4	19.5	17.8	10.1	11.0	2.8	1.2
EPB	0.25-512	99.0±8.2	103.2±2.6	88±6.7	12.7	10.3	13.1	8.0	6.0	1.5
iso-PPB	0.25-512	75.9±11.5	70.7±6.1	69±8.0	19.9	0.1	3.9	7.0	4.7	1.9
nPPB	0.25-512	89.8±4.6	95.4±4.3	86±6.9	5.1	3.9	3.7	15.0	1.4	3.4
iso-BPB	0.25-512	75.3±5.1	80.8±3.8	77±5.7	8.6	6.5	7.5	9.3	1.9	1.8
nBPB	0.25-512	69.8±4.8	87.7±2.7	83±5.6	15.2	1.1	1.1	8.6	1.8	1.5
BPA (dansylated)	0.05-64	75.2±4.2	78.9±4.3	88±5.6	3.1	1.9	1.4	2.4	2.6	1.6

SD: standard deviation

3.5 Concentration levels in urine

Concerning about the mother-child pairs (pregnancy-2.5 y.o.) levels, the metabolites mEP, mnBP, miBP, mEOHP, mEHHP, mBzP , MPB, EPB, nPPB and BPA were detected in >90.8% of mother samples (Table 7) and in >86.2% of children samples (Table 8), while mEHP was detected in the 72.7 % of mother and 57.3% of children samples; isoPPB, isoBPB and nPPB ranged from 12.0% to 38.6% detectability for mothers (Table 9) and 2.1% to 25.9% for children (Table 10). mNP was not detected in any of the analysed samples, thus we do not consider it as a proper urine biomarker for DiNP exposure. Secondary metabolites, (e.g. mCOP, mono-

carboxy-octyl phthalate) of this PE should be used as biomarkers. The same conclusions concerning mNP were also drawn in other studies (Koch et al., 2007; Silva et al., 2006b). For this reason, mNP determination was sont prefromed in 4-years old children. Due to the variable density of urine spot samples (Barr et al., 2005b), we preferred to use creatinine normalisation in order to rank the concentration levels of studied compounds.

Among the creatinine adjusted median levels of PEs metabolites, mEP was the most abundant for mothers (133.6 µg/g) and miBP for children (101.6 µg/g); miBP concentrations were in higher levels compared to mnBP for both categories; from DEHP metabolites, mEHHP and mEOHP were in higher levels compared to mEHP also for both categories; mBzP was the less abundant detected PEs metabolite (Tables 7 and 8). Concerning creatinine adjusted median PBs levels, MPB was the most abundant for both categories (mothers: 121.9 µg/g, children: 42.6 µg/g), but for mothers, nPPB levels were higher compared to EPB and for children EPB levels were higher to nPPB; the other three PBs had median values < mLOD. BPA levels were found at 1.1 µg/g for mothers and at 5.2 µg/g for children. (Table 9 and 10).

Concerning about 4 years old children, PEs metabolites levels for both unadjusted and creatinine adjusted concentrations are presented in Table 11 while PBs and BPA levels are depicted in Table 12. Detectability of PEs metabolites were >93.4%. DEHP metabolites and mEP were detected in all samples. MPB, EPB and nPPB were below mLOD at >92.6%. nBPB was detected in 37.6% of the samples while isoPPB and isoBPB were detected with lower rate (3.8% and 10% respectively). Finally, BPA detected in almost all samples (98.8%). Based on creatinine adjusted median concentration levels, mEP was the most abundant PEs metabolite followed in decreasing order by miBP, mEHHP, mEOHP, mnBP, mEHP and mBzP (Table 11). Furthermore, concerning about PBs, creatinine normalised median levels of MPB were the highest (17.6 µg/g) among the examined PBs, EPB and nPPB were in the same levels (1.5 µg/g). The other three PBs, when we examined their creatinine adjusted arithmetic mean (their detectability were <50% therefore median could not be calculated) levels showed that the most abundant was nBPB (1.0 µg/g) followed by isoBPB and isoPPB (0.2 and 0.1 µg/g respectively). BPA creatinine adjusted median levels were at 1.9 µg/g (Table 12).

When 2.5 year old children were categorized by gender, males exhibited, statistically significant (Mann-Whitney U test; p-value<0.05), higher unnormalised concentrations (ng/mL) for nPPB and all PEs metabolites except from mEHP (Table 11). We used unnormalised concentrations because creatinine levels were higher in males (arithetic mean, males: 0.53 g/L, females: 0.45 g/L; independent samples t-test, p-value: 0.043). Independent samples t-test was utilised to compare creatinine levels since creatinine data were not skewed. No gender-based differentiations were observedin 4 years old children. Analogous studies show controversial reuslts about relation of gender with children with metabolite levels, a fact which denotes influence of more parameters, for e.g. age (Boas et al., 2010; Cutanda et al., 2014; Guo et al., 2011; Langer et al., 2014; Song et al., 2013; Wittassek et al., 2007; Zhang et al., 2014).

Table 7. Pregnancy: descriptive statistics of PEs metabolites urinary concentrations, ng/mL urine (μg/g creatinine)

PEs metabolite	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	mEHP
Detected (%>mLOD)	100.0	98.0	95.9	96.4	93.6	91.6	72.7
Minimum	2.6	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD
	(4.8)						
Median	133.9	39.2	36.1	25.7	17.6	6.0	7.6
	(132.6)	(38.7)	(33.2)	(24.4)	(15.9)	(7.0)	(7.3)
95th percentile	1462.9	189.4	210.5	125.5	100.5	38.2	50.1
	(1230.9)	(131.9)	(157.1)	(107.8)	(84.8)	(32.1)	(47.1)
Maximum	4103.7	616.1	94670.7	6267.3	3610.6	199.4	3401.3
	(3993.8)	(720.0)	(48799.3)	(5095.4)	(2935.4)	(132.0)	(2765.3)
Arithmetic mean	360.9	62.0	463.9	66.3	49.4	12.8	28.2
	(323.2)	(54.7)	(260.2)	(60.5)	(42.6)	(11.2)	(25.3)
Geometric mean	141.9	36.7	32.1	22.1	15.5	6.9	7.0
	(143.5)	(37.1)	(32.5)	(22.3)	(15.7)	(7.0)	(7.1)
Geometric Mean 95% CI	119.3-171.8	32.2-42.3	27.3-38.5	18.9-26.1	13.1-18.5	6.1-8.0	6.0-8.2
	(122.0-169.8)	(33.2-41.6)	(28.4-37.5)	(19.5-26.1)	(13.7-18.5)	(6.2-7.9)	(6.1-8.3)

Table 8. 2.5 years old children: descriptive statistics of PEs metabolites urinary concentrations, ng/mL urine (μg/g creatinine)

PEs metabolite	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	mEHP
Detected (%>mLOD)	99.6	98.7	96.2	97.1	95.4	86.2	57.3
Minimum	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD
Median	34.4	34.4	23.9	30.5	20.0	6.5	2.8
	(86.6)	(101.6)	(62.3)	(71.0)	(51.0)	(17.0)	(9.1)
95th percentile	230.4	202.4	162.3	158.2	116	35.2	23.5
	(477.7)	(280.6)	(261.0)	(246.7)	(171.6)	(73.5)	(78.5)
Maximum	2460.1	886.0	1250.5	626.3	391.1	241.9	95.01
	(3617.8)	(681.6)	(962.0)	(1204.3)	(611.1)	(394.2)	(203.8)
Arithmetic mean	79.2	62.4	47.1	51.2	35.0	12.9	7.0
	(182.7)	(123.2)	(90.8)	(102.2)	(68.4)	(27.8)	(18.2)
Geometric mean	35.3	36.0	23.3	24.9	16.9	6.8	3.8
	(88.5)	(90.3)	(58.3)	(62.4)	(42.5)	(17.0)	(9.6)
Geometric Mean 95% CI	30.2-41.0	31.2-41.4	19.9-27.0	20.8-29.7	14.2-20.5	5.9-7.8	3.4-4.4
	(76.9-102.0)	(81.0-100.8)	(51.5-65.9)	(54.3-71.9)	(37.3-48.9)	(15.0-19.3)	(8.5-11.0)

Table 9. Pregnancy: descriptive statistics of PBs and BPA urinary concentrations, ng/mL urine (μg/g creatinine)

Phenol	MPB	EPB	isoPPB	nPPB	isoBPB	nBPB	BPA
Detected (%>mLOD)	99.2	93.6	12.0	90.8	26.5	38.6	99.6
Minimum	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD
Median	98.3 (121.9)	2.6 (2.9)	<mLOD	13.4 (17.5)	<mLOD	<mLOD	1.2 (1.1)
95th percentile	3098.4 (3191.5)	120.5 (77.3)	0.97 (0.85)	685.4 (461.4)	2.6 (2.3)	28.2 (21.3)	4.7 (5.6)
Maximum	67461.3 (46089.3)	377.5 (146.3)	63.9 (51.1)	28182.1 (20387.3)	59.2 (39.2)	242.3 (148.8)	144.0 (116.1)
Arithmetic mean	1200.7 (1138.8)	19.8 (16.0)	0.9 (0.9)	413.4 (365.7)	0.6 (0.5)	6.2 (5.1)	2.6 (2.4)
Geometric mean	102.1 (103.2)	3.1 (3.2)	NC	11.2 (11.3)	NC	NC	1.2 (1.2)
Geometric Mean 95% CI	79.8-132.8 (80.5-132.6)	2.5-4.1 (2.5-4.1)	NC	8.0-15.4 (8.1-15.4)	NC	NC	1.1-1.4 (1.1-1.4)

Table 10. 2.5 years old children: descriptive statistics of PBs and BPA urinary concentrations, ng/mL urine (μg/g creatinine)

Phenol	MPB	EPB	isoPPB	nPPB	isoBPB	nBPB	BPA
Detected (%>mLOD)	100.0	93.3	2.1	79.1	6.3	25.9	99.6
Minimum	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD
Median	17.1 (42.6)	1.5 (3.7)	<mLOD	0.9 (2.3)	<mLOD	<mLOD	2.1 (5.2)
95th percentile	942.9 (2710.5)	96.2 (318.2)	<mLOD	111.7 (328.5)	0.1 (0.4)	1.3 (2.2)	16.6 (31.0)
Maximum	6805.9 (17014.8)	1116.9 (1801.5)	10.8 (13.0)	1491.3 (3728.3)	1.1 (1.7)	93.2 (665.9)	68.7 (121.7)
Arithmetic mean	198.2 (512.2)	22.0 (66.0)	0.1 (0.4)	25.7 (73.1)	0.1 (0.1)	1.0 (5.9)	4.5 (9.6)
Geometric mean	25.0 (62.7)	1.8 (4.5)	NC	1.3 (3.2)	NC	NC	2.0 (5.0)
Geometric Mean 95% CI	20.0-31.5 (49.5-79.7)	1.4-2.4 (3.4-6.0)	NC	0.9-1.7 (2.4-4.4)	NC	NC	1.7-2.4 (4.3-5.8)

Table 11. 4 years old children: descriptive statistics of PEs metabolite levels, ng/mL urine (µg/g creatinine)

PEs metabolite	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	mEHP
Detected (%>mLOD)	100.0	96.8	93.4	100.0	100.0	99.0	100.0
Arithmetic mean	131.6 (159.5)	43.3 (62.5)	32.7 (45.3)	44.3 (67.4)	36.4 (54.5)	10.1 (14.3)	10.6 (17.2)
Minimum	1.0 (0.9)	<mLOD	<mLOD	0.2 (0.2)	0.4 (0.4)	<mLOD	1.1 (1.7)
Median	34.9 (53.5)	29.5 (48.8)	17.2 (27.9)	27.4 (40.7)	22.6 (35.4)	4.5 (7.0)	6.2 (10.5)
95th percentile	293.1 (416.0)	130.5 (158.2)	104.4 (128.8)	124.5 (161.7)	103.7 (124.5)	35.3 (50.6)	34.4 (46.5)
Maximum	19549.1 (17611.8)	586.3 (671.4)	564.4 (695.0)	1504.9 (2246.1)	1107.3 (1652.7)	426.5 (313.6)	141.1 (300.7)
Geometric Mean	38.0 (62.7)	24.8 (40.9)	13.1 (21.6)	26.3 (43.3)	21.0 (34.6)	4.4 (7.3)	6.7 (11.1)
Geometric Mean 95% CI	34.1-42.3 (57.1-68.9)	22.2-27.8 (36.9-45.4)	11.3-15.2 (18.9-24.7)	24.0-28.7 (40.3-46.6)	19.1-23.0 (32.0-37.3)	4.0-5.0 (6.6-8.1)	6.2-7.3 (10.3-11.9)

Table 12. 4 years old children: descriptive statistics of PBs and BPA levels, ng/mL urine (µg/g creatinine)

Phenol	MPB	EPB	iso PPB	nPPB	isoBPB	nBPB	BPA
Detected (%>mLOD)	100.0	96.8	3.8	92.6	10.0	37.6	98.8
Arithmetic mean	79.6 (147.2)	7.7 (12.0)	0.1 (0.1)	10.0 (17.2)	0.2 (0.2)	0.6 (1.0)	2.0 (3.2)
Minimum	0.7 (0.8)	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD
Median	11.5 (17.6)	0.9 (1.5)	<mLOD	0.9 (1.5)	<mLOD	<mLOD	1.2 (1.9)
95th percentile	263.3 (424.7)	25.5 (43.5)	<mLOD	34.3 (49.3)	0.1 (0.2)	0.7 (1.4)	6.4 (10.0)
Maximum	3846.7 (11039.7)	315.2 (573.1)	15.0 (14.5)	483.2 (1098.1)	50.4 (45.4)	97.1 (158.9)	59.2 (67.3)
Geometric Mean	15.1 (24.9)	1.1 (1.9)	NC	1.1 (1.9)	NC	NC	1.1 (1.7)
Geometric Mean 95% CI	13.3-17.2 (22.0-28.3)	1.0-1.3 (1.6-2.1)	NC	1.0-1.3 (1.6-2.2)	NC	NC	1.0-1.2 (1.6-1.9)

The overall levels of all studied metabolites are relatively lower in 4 years old children compared with 2.5 years old (Tables 8 and 10-12). Furthermore levels in all three population groups are generally comparable

with the literature reports from other countries. The overall PEs exposure, evaluated in our study, is generally comparable with the literature reports from other countries. Furthermore, the specific PEs exposure pattern is also similar in general (Tables 14 and 15). Comparison with other studies demonstrated slightly higher exposure to PEs for mothers in Greece compared to those from Denmark (Tefre de Renzy-Martin et al., 2014) and slightly lower compared to those from France (Mortamais et al., 2012; Zeman et al., 2013) (Table14). Moreover, for children there are not distinct differences for PEs metabolites levels in general (Table 15). However, our study exhibited the highest miBP and generally low mnBP levels. This fact may be explained by the gradual replacement of DnBP (parent compound of mnBP) with DiBP (parent compound of miBP) (Table 1) and the point that our study is the most recent. Concerning about PBs, although concentration levels were lower at 4-year children compared to 2.5-year, in both cases they appeared to be clearly higher compared to a study from Denmark (Frederiksen et al., 2013). Finally, BPA levels were average in relation to the other available reports (Table 4).

Table 13. Gender-based comparison of 2.5 years old children arithmetic mean concentration levels (ng/mL)

	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	nPPB
Male	87.88	67.41	46.29	57.92	37.02	15.08	30.26
Female	67.79	55.82	48.27	42.24	32.31	10.14	19.66
p-value	0.006	0.004	0.011	0.005	0.015	0.009	0.036

It has to be mentioned that bibliographic comparisons suffer from several weaknesses as they don't take into account, market changes of PEs, PBs and BPA, corresponding to different time periods of sample collection, different age of children and phase of pregnancy, different methods of analysis and analyte detection limits, lack of statistical tests for inter-study comparisons etc. Especially, for PBs levels, more data are needed in order to assess globally their exposure.

Table 14. Pregnant women: comparison with similar studies, creatinine adjusted median (μg/g) values

Country; number of samples; reference	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	mEHP	MPB	EPB	isoPPB	nPPB	isoBPB	nBPB	BPA
Greece; n=239; this study	132.6	38.7	33.2	24.4	15.9	7.0	7.3	121.9	2.9	<LOD	17.5	<LOD	<LOD	1.1
France; n=287; (Mortamais et al., 2012)	106.0	45.7	48.5	-	-	16.0	-	-	-	-	-	-	-	-
Denmark; n=200; (Tefre de Renzy-Martin et al., 2014)	18.9	35.3	13.9	5.7	3.72	2.3	1.1	-	-	-	-	-	-	-
France; n=279; (Zeman et al., 2013)	34.3	68.7	45.5	44.4	32.9	13.0	17.9	-	-	-	-	-	-	-
Denmark; n=143; (Frederiksen et al., 2013)	-	-	-	-	-	-	-	16.0	0.91	<LOD	1.8	<LOD	<LOD	-
Korea; n=757; (Lee et al., 2014)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6
USA; n=866; (Quiros-Alcala et al., 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1
Spain; n=479 (Casas et al., 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2
USA; n=244; (Braun et al., 2011)	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2
USA; n=249; (Braun et al., 2009)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8

Table 15. Children: Comparison with similar studies, creatinine adjusted median (µg/g) values

Country; number of samples; age (y); reference	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	mEHP	MPB	EPB	isoPPB	nPPB	isoBPB	nBPB	BPA
Present study; Greece; n=500	53.5	48.8	27.9	40.7	35.4	7.0	10.6	17.6	1.5	<mLOD	1.5	<mLOD	<mLOD	1.9
Previous Rhea study, 2.5-year children; Greece; n=239;	86.6	101.6	62.3	71.0	51.0	17.0	9.1	42.6	3.7	<LOD	2.3	<LOD	<LOD	5.2
Denmark; n=845; 4-9 y; (Boas et al., 2010)	33.5	-	209.0	52.0	27.0	23.0	6.8	-	-	-	-	-	-	-
USA; n=379; 7.3 y; (Teitelbaum et al., 2012)	164.9	22.5	68.4	73.9	47.6	41.8	6.4	-	-	-	-	-	-	-
Germany; n=465; 8-10 y; (Kasper-Sonnenberg et al., 2014)	21.4	41.1	42.3	20.2	13.5	6.0	2.23	-	-	-	-	-	-	1.8
Denmark; n=143; 6-11 y; (Frederiksen et al., 2013)	-	-	-	-	-	-	-	0.9	0.26	<LOD	<LOD	<LOD	<LOD	-
USA; n=292; 5 y; (Harley et al., 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	3.2
China; n=1089; 9 y; (Hong et al., 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.3
China; n=666; 9-12 y; (Wang et al., 2014)	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2
USA; n=229; 1-3 y; (Braun et al., 2011)	-	-	-	-	-	-	-	-	-	-	-	-	-	14.0

3.6 DEHP metabolism

Relative metabolic rates RMR_1 and RMR_2 were calculated for mothers (N=170), 2.5 years old children (total N=136, female and male) and 4 years old children (N=500) for samples in which, all three DEHP metabolites were detected. The RMR_1 arithmetic mean for mothers was 3.33, for 2.5 years old children 8.06 and for 4 y.o. 5.11. Male 2.5 y.o. children mean RMR_1 (8.83) was higher from 2.5 y.o. female children (6.67) but without statistically significant difference (independent sample t-test, p-value 0.17). Instead, male 2.5 y.o. children mean RMR_2 (0.72) was significantly lower (p-value 0.03) compared to 2.5 y.o. female children (0.81). RMR_2 at 4 y.o. children arithmetic mean was 0.85. We compared RMRs in 4-year children with 2.5-year. We didn't not compared with pregnant women due to heterogeneity among population groups. The results are depicted in Figure 4. The RMR_1 in 4-year children is statistically significantly lower (independent samples t-test, p-value<0.001) compared to 2.5-year children (RMR_1: 8.06). RMR_2 was in comparable levels for 4-year (0.85) and 2.5-year (0.76) children.

To sum up: I) the transformation of mEHHP to mEOHP seems to be faster in 2.5 y.o. male children compared to female and II) the transformation of mEHP to mEHHP (as expressed by RMR_1) seems to be negatively related with age, an observation also reported by Song et al. (Song et al., 2013). However, other studies (Barr et al., 2003; Becker et al., 2004; Kasper-Sonnenberg et al., 2012; Koch et al., 2004b) reported contrasting results, indicating that DEHP metabolism is related both with children age/gender and differentiates between mothers and children.
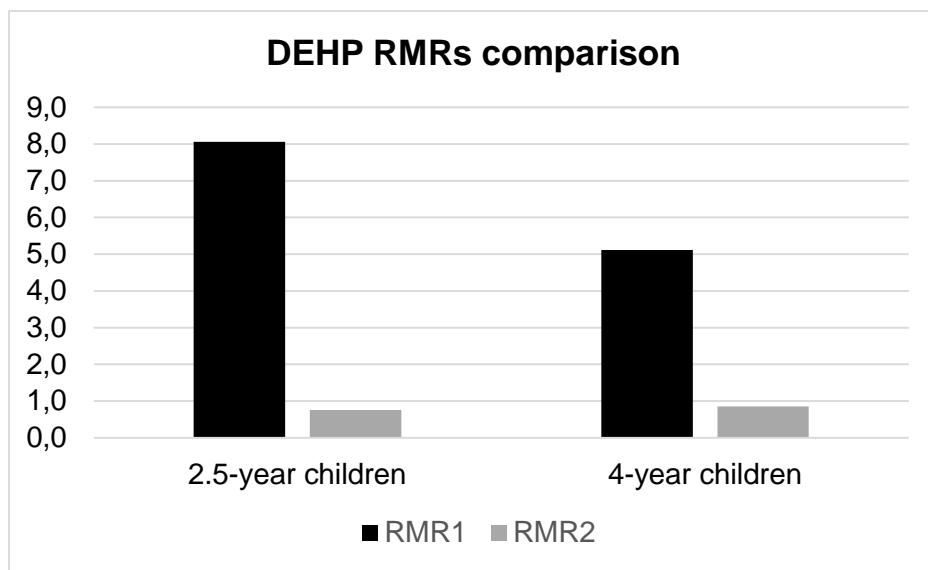

Figure 4. DEHP relative metabolic rates comparison, arithmetic mean values

3.7 Patterns of exposure

Statistically significant correlations (2-tailed Spearman) were observed between the creatinine adjusted molar concentrations (μmol/g) of the studied compounds both for mothers and children. We only

present correlations with p-values lower than 0.01. Almost all studied metabolites correlated positively (Tables 16 and 17). The observed correlations suggest exposure to mixtures of PBs, PEs and BPA, although further analysis is required to trace more specific mixtures. 2.5 y.o. children exact age correlated negatively (-0.193 - -0.476) with all studied metabolites concentration levels (Table 17). An interpretation of this observation is that the food exposure ratios (consuming food mass/body mass), the corresponding dermal exposure ratios (skin surface/body mass), and the floor-to-mouth behaviour decrease as children age increases (Casas et al., 2011; Wittassek et al., 2007). For 4 y.o. children, negative correlation with age was observed only for BPA (-0.176, p-value<0.01) possibly due to the lower age variance of the 4 y.o. children and the lower development rate in higher ages. Finally, DEP, DnBP, BBP metabolites and EPB showed weak (0.133-0.225) correlation between mother and 2.5 y.o. children levels, as also observed previously (Cutanda et al., 2014). No statistically significant correlations were found for the remaining studied compounds between mothers and children (Table 17). This fact indicates that, although we examined two different individuals within a time interval of ca. three years (sample collection time from women during pregnancy and from their children) implying also changes in PEs-PBs-BPA market, some sources of exposure are common for people living together (house, car use etc.). Furthermore, the need for repeated measurements is highlighted since the correlation between mother and children levels is weak.

Table 16. Two-tailed Spearman's correlation coefficients, p-values <0.01

	DiBP	DnBP	BBP	DEHP	MPB	EPB	nPPB	BPA
Age (y)								-0.176
DEP	0.463	0.333	0.332	0.494	0.453	0.367	0.409	0.274
DiBP		0.685	0.634	0.614	0.371	0.307	0.387	0.447
DnBP			0.699	0.629	0.310	0.268	0.334	0.508
BBP				0.637	0.292	0.247	0.349	0.395
DEHP					0.395	0.343	0.411	0.454
MPB						0.620	0.675	0.273
EPB							0.569	0.301
nPPB								0.293

In order to obtain more specific information about exposure patterns, a PCA was applied to concentration data. The results were identical for all three population groups (see Appendix 2 and 3). For this reason, they are presented from 4 years old children (see Appendix 2 and 3 for 2.5 y.o. children and pregnant women respectively). Two distinct patterns were perceived (Table 18, Figure 5), one is mostly due to usage of plastics such as food packaging, toys, car parts, clothing, furniture etc. and the second is usage of personal care-hygiene products and cosmetics (Elder 1984; Geens et al., 2012; Wormuth et al., 2006). Two factors were retained with Eigen values over 1.000 and expressed 54.96% of the variance. The first factor indicates that PBs exposure is combined furthermore DEP is present in these mixtures since all PBs and DEP are

correlated positively. The second factor denotes that BPA and PEs are correlated and the exposure to them is also combined. The PBs-DEP mixtures are possibly from usage of personal care-hygiene products and the PEs-BPA from plastic usage (food packaging, toys, car parts, clothing, furniture etc.) (Elder, 1984; Geens et al., 2012; Wormuth et al., 2006). The same distinct patterns were also perceived in 2.5-year children and during pregnancy.

Table 17. Mother-child pairs: two-tailed Spearman's correlation coefficients, p-values <0.01

	M-DnBP	M-BBzP	M-DEHP	M-MPB	M-EPB	M-nPPB	M-BPA	C-DEP	C-DiBP	C-DnBP	C-BBzP	C-DEHP	C-MPB	C-EPB	C-nPPB	C-BPA
M-DEP			0.17	0.25	0.34	0.28		0.20								
M-DiBP	0.44	0.35	0.42													
M-DnBP		0.37	0.49							0.23						
M-BBzP			0.44				0.25				0.18					
M-MPB					0.54	0.84										
M-EPB						0.46								0.13		
C-Age (y)								-0.43	-0.43	-0.48	-0.37	-0.19	-0.38	-0.47	-0.38	-0.21
C-DEP									0.52	0.48	0.38	0.27	0.36	0.40	0.37	0.19
C-DiBP										0.76	0.44	0.42	0.32	0.31	0.34	0.29
C-DnBP											0.58	0.48	0.29	0.34	0.34	0.31
C-BBzP												0.50	0.27	0.31	0.27	0.32
C-DEHP													0.17		0.19	0.30
C-MPB														0.67	0.81	
C-EPB															0.67	0.20

M-: mothers, C-: children
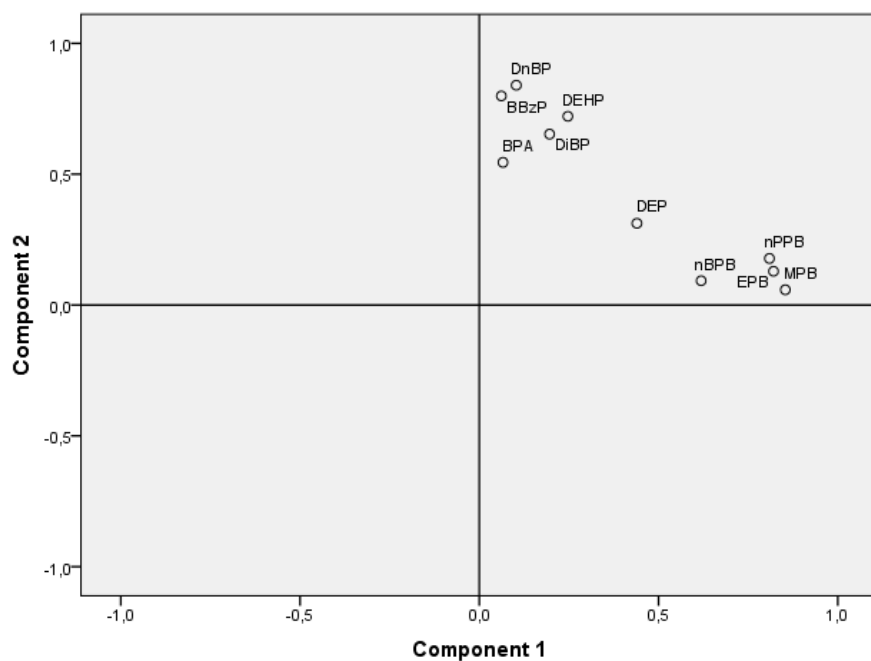
Figure 5. 4 years old children: principal components plot

Table 18. 4 years old children: rotated component matrix and total variance explained of PCA

Component	1	2
Eigenvalue	3.72	1.78
% of Variance	37.20	17.76
Cumulative %	54.96	37.20
DnBP	0.10	0.84
BBP		0.80
DEHP	0.25	0.72
DiBP	0.20	0.65
BPA		0.55
DEP	0.44	0.31
MPB	0.85	
EPB	0.82	0.13
nPPB	0.81	0.18
nBPB	0.62	

Coefficients <0.1 are not presented. Rotation converged in 3 iterations

3.8 Estimated daily intake based on urinary metabolites, indoor air and drinking water concentration levels

The estimated daily intake levels for all PBs and PEs (median values, Table 19) were higher for mothers in pregnancy than for their children at 2.5 years of age (Wilcoxon signed ranked test, p-value<0.001), except for DEHP (also higher in mothers but p-value was 0.055), as shown in Figure 6. In contrast, BPA-DI$_u$ was higher in children compared to their mothers (Wilcoxon signed ranked test, p-value 0.013). Although, we must take into account that there is an almost three years distance between samplings and except for differentiations in pregnant-child exposure, changes in PEs, PBs and BPA market may also affect exposure levels. DEP for mothers and DEHP for children, were the two PEs with the highest median values of DI$_u$, followed by DiBP, DnBP and BBP in decreasing order. The estimated DI$_u$ of all PEs was higher in mothers compared to children, in contrast to the creatinine-normalised values (Tables 7-12). This is explained by the fact that although creatinine adjustment is a very useful tool to normalise urine density and compare urinary concentration of a homogenous population, it does not take into account that creatinine excretion is dependent on muscle mass and body height (Barr et al., 2005b). Furthermore, DEHP DI$_u$ was the highest in children and the second highest in mothers, in contrast to DEHP metabolites levels, which were relatively lower compared to those of other PEs metabolites. This observation is due to the extended metabolism of DEHP to many compounds (Koch et al., 2005b). The values of Reference Doses (RfD, µg d^{-1} kg^{-1}; DEP: 800, DnBP: 100, BBP: 200, DEHP: 20, BPA: 50) established by U.S. Environmental Protection Agency (U.S. Environmental Protection Agency, 2005a; U.S. Environmental Protection Agency, 2005b; U.S. Environmental Protection Agency, 2005c) and of Tolerable Daily Intake (TDI, µg d^{-1} kg^{-1}; DEP:500, DnBP:10, BBP:500, DEHP:50, BPA: 50 (established) / 5 (temporary)) set by the European Food Safety Authority (European Food Safety Administration, 2005), were compared with our results. Concerning about mother-child pairs (pregnancy-2.5 y.o.), for DnBP, the 5.9% of mothers revealed higher DnBP-DI$_u$ than DnBP-TDI (0.8% of subjects higher compared to DnBP-RfD). For DEHP, the 6.8% of mother's DI$_u$ exceeded RfD (0.6% exceeded DEHP-TDI). For 2.5 y.o. children, the 1.7% of cases were found exceeding DnBP-TDI. Finally, the 6.3% of 2.5 y.o. children's DEHP-DI$_u$ was higher than the corresponding DEHP-RfD (1.3% of subjects exceeded TDI). For PBs daily intake, the same patterns were observed with concentration values (Tables 3 and 4, Figure 1). The higher intake of PBs and PEs (except from DEHP) by mothers can be interpreted by the extensive usage of cosmetics (Elder 1984). This was not observed for DEHP, which is widely used as plasticizer (Wormuth et al., 2006). BPA-DI$_u$ was the lowest among the examined endcrine disruptors for both mothers and children. Furthemore, none subject of the study exceeded neither current nor recommended BPA-TDI (current: 50 µg d^{-1} kg^{-1} / recommended: 4 µg d^{-1} kg^{-1}) (EFSA 2014).

Concerning about 4 years old children (Table 20) highest median PEs-DI$_u$ was exhibited by DEHP (4.02 µg d^{-1} kg^{-1}) followed by DnBP, DiBP and DEP (0.70 – 1.30 µg d^{-1} kg^{-1}). The DI$_u$ of BBP was in relatively lower levels (0.17 µg d^{-1} kg^{-1}). Regarding about PBs, MPB had the highest DI$_u$ (25.75 µg d^{-1} kg^{-1}) with EPB and nPPB at comparable levels (1.93 - 2.01 µg d^{-1} kg^{-1}). BPA showed the lowest median DI$_u$ (0.026 µg d^{-1} kg^{-1}) (Table 5). For RfD, 3.6% of the children exceeded DEHP-RfD (20 µg d^{-1} kg^{-1}). For TDI, 0.2% exceeded DEP-TDI, 0.4% DiBP-TDI, 1% exceeded DnBP-TDI and 1% DEHP-TDI. BPA-DI didn't exceed even the strictest proposed-TDI (4 µg d^{-1} kg^{-1}) in any case.

Table 19. Pregnant women-their 2.5 tears old children: estimated daily intake of PEs, PBs and BPA

	Minimum	Median	95th percentile	Maximum	Arithmetic mean	Geometric mean	95% CI
239 mothers, (μg d^{-1} kg^{-1})							
DEP	0.2	6.9	74	182.4	17.9	7.1	6.0-8.4
DiBP	NC	2.1	11	30.6	3.4	2	1.7-2.3
DnBP	NC	1.9	11.4	4839.8	24	1.7	1.5-2.1
DEHP	NC	4.4	25.6	1015.0	12.2	4.1	3.5-4.8
BBzP	NC	0.3	1.8	9.9	0.6	0.3	0.3-0.4
MPB	NC	500.0	17076.1	388041.8	6388.1	532.7	413.6-688.8
EPB	NC	13.2	599.9	2388.4	109.3	16.4	12.4-21.3
isoPPB	NC	NC	4.0	281.2	4.8	NC	NC
nPPB	NC	73.3	3818.5	162105.5	2203.1	58.2	40.1-82.2
isoBPB	NC	NC	8.1	183.5	2.0	NC	NC
nBPB	NC	NC	82.6	781.4	20.0	NC	NC
BPA	NC	0.03	0.14	4.23	0.08	0.04	0.03-0.04
239 2.5 years old children, (μg d^{-1} kg^{-1})							
DEP	NC	1.4	8.6	91.4	2.9	1.3	1.1-1.5
DiBP	NC	1.4	8.2	36.0	2.5	1.5	1.30-1.7
DnBP	NC	1.0	6.6	50.8	1.9	0.9	0.8-1.1
DEHP	NC	4.0	21.6	69.6	6.8	3.3	2.8-3.9
BBzP	NC	0.2	1.3	9.0	0.5	0.3	0.2-0.3
MPB	NC	66.6	3674.9	26526.7	772.5	97.5	77.5-123.54
EPB	NC	5.8	375.1	4353.3	85.9	7.0	5.4-9.1
isoPPB	NC	NC	NC	42.0	0.5	NC	NC
nPPB	NC	3.4	435.4	5812.6	100.1	5.0	3.7-6.7
isoBPB	NC	NC	0.16	2.6	0.1	NC	NC
nBPB	NC	NC	2.9	217.0	2.3	NC	NC
BPA	NC	0.05	0.37	1.54	0.10	0.04	0.04-0.05

NC: not calculated

DI$_u$ levels for 4-years old children were compared with those for 2.5-years old children as individual populations, using Mann-Whitney U test. As can be seen in Figure 6, 4-year old children displayed the lowest DI$_u$ for all examined chemicals (Tables 19 and 20). This difference is lower vs 2.5-year old children for all studied compounds (p-value < 0.003) except DEP and DEHP. We tested also the differences between only the paired samples at 2.5-year and 4-year (Wilcoxon signed ranked test) and the results were similar. To conclude, there is general trend in most cases which shows that DI$_u$ is higher in 2.5-years old children compared to 4-years old. This trend aids the fact that early childhood is the most vulnerable life period to

endocrine disruptor exposure (World Health Organization, 2012) due to higher exposure except from increased human organism susceptibility. Although, it must be taken into account the changes/restrictions in PEs, BPA (European Food Safety Administration, 2015) and PBs market probably have lowered/altered the exposure through last years and therefore, between sampling periods.

Table 20. Estimated daily intake of PEs, PBs and BPA ($\mu g \ d^{-1} \ kg^{-1}$)

	Arithmetic mean	Geometric mean	Geometric mean 95% CI	Minimum	Median	95% percentile	Maximum
DEP	4.89	1.41	1.27-1.57	0.04	1.30	10.89	726.23
DiBP	1.76	1.01	0.90-1.13	<mLOD	1.20	5.31	23.84
DnBP	1.33	0.53	0.46-0.62	<mLOD	0.70	4.25	22.94
BBP	0.38	0.17	0.15-0.19	<mLOD	0.17	1.32	15.95
DEHP	6.48	3.83	3.50-4.18	0.06	4.02	17.30	206.92
MPB	178.34	33.88	29.78-38.54	1.52	25.75	589.88	8616.56
EPB	17.15	2.52	2.18-2.91	<mLOD	2.01	57.23	706.04
isoPPB	0.32	NC	NC	<mLOD	<mLOD	<mLOD	33.50
nPPB	22.41	2.55	2.16-3.00	<mLOD	1.93	76.74	1082.29
isoBPB	0.54	NC	NC	<mLOD	<mLOD	0.31	112.85
nBPB	1.43	NC	NC	<mLOD	<mLOD	1.48	217.56
BPA	0.045	0.024	0.021	<mLOD	0.026	0.143	1.327

NC: not calculated

Table 21. Concentration levels of PEs in indoor air and tap water and corresponding daily intake compared with urinary daily intake, average values.

PEs	Home air concentration (ng/m^3)	Car interior air concentration (ng/m^3)	Tap water concentration pg/mL	Air daily intake (DI$_a$) ($\mu g \ d^{-1} \ kg^{-1}$)	Water daily intake (DI$_w$) ($\mu g \ d^{-1} \ kg^{-1}$)	Total daily intake 4-year old children (DI$_u$) ($\mu g \ d^{-1} \ kg^{-1}$)
DEP	1705.1	2459.6	574.6	0.733	0.031	4.888
DiBP	944.6	759.5	2058.6	0.405	0.112	1.762
DnBP	467.6	235.8	1165.4	0.200	0.063	1.329
BBP	5.8	7.3	<LOD	0.002	NC	0.379
DEHP	178.2	350.4	415.9	0.077	0.023	6.475

NC: not calculated

PEs concentration levels in water, indoor air (homes and cars) and the estimated corresponding daily intakes (from water, air and from all sources calculated through urine concentrations) are presented in Table

21. Home and car air showed comparable concentration patterns, with DEP being the most abundant PE, followed by DiBP, DnBP/DEHP and BBP. For drinking water, DiBP showed the highest levels followed by DnBP, DEP and DEHP. BBP was not detected in water samples. The estimated DI_w and DI_a indicated that intake though air inhalation is generally higher compared to water ingestion. However, both of these routes of exposure represent a small fraction of the total PEs exposure, expressed by DI_u. Although the different periods of sampling for air, water and urine may limit the accuracy of our estimations, we consider that the differences in DI levels are very important to contradict our observations.
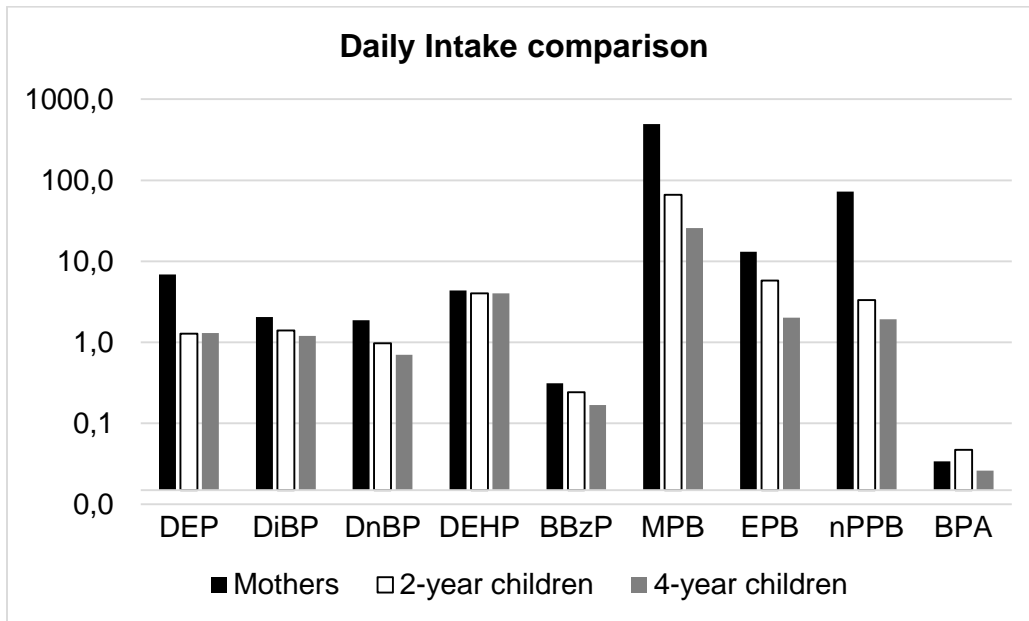

Figure 6. Estimated daily intake comparison for Rhea cohort subjects, median values ($\mu g\ d^{-1}\ kg^{-1}$), y axis in logarithmic scale

4. Conclusions

We have developed the first common clean-up procedure for the determination of phthalate metabolites, PBs and BPA. In addition, we elaborated an HPLC-ESI-MS/MS method, with chromatographic characteristics, suitable for the identification and quantification of seven phthalate metabolites, six PBs and BPA in human urine. This method provided a clear separation of n-/iso- structural isomers of butyl-paraben and propyl-paraben. To the best of our knowledge this is reported for the first time by using conventional HPLC columns. The described clean-up procedure and LC/MS method were successfully applied to human urine analysis allowing the determination of the reported analytes in a large number of samples.

Furthermore, we assessed the exposure levels of PEs, PBs and BPA in two hundred and thirty nine pregnant women, in their children at 2.5 years of age and in five hundred (500) 4-year old children (all subjects of Rhea cohort). In our study, the first in Greece and one of the few existing in this scale globally, we observed lower phthalate (except DEHP) and PBs daily intake for children than mothers while BPA daily intake was higher in children. Total daily intake at 4-year old children was lower than the corresponding of 2.5-year old children. The determination of PEs in drinking water and indoor air, in order to evaluate their corresponding exposure, revealed their low input to the total exposure, evaluated through the metabolites urine concentrations. Comparison with other studies worldwide did not reveal strong differentiations in concentration levels for PEs metabolites and BPA. For PBs there is need for more studies to proceed to solid conclusions. For DiNP exposure, mNP is not a proper biomarker. Children metabolite levels decreases with age increase. Male 2.5 years old children demonstrated higher concentrations of six PEw metabolites and n-PPB compared to females at the same age. PCA grouped the exposure to two distinct sources: plastic for PEs-BPA and personal care-hygiene products for PBs-DEP. DEHP metabolism rate appears differentiated between mother-child pairs and female-male children. The rate of mEHP transformation to mEHHP (a phase of DEHP metabolism) seems to be age- and sex- related.

5. References

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Appendix 1

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Title: Determination and separation of bisphenol A, phthalate metabolites and structural isomers of parabens in human urine with conventional high-pressure liquid chromatography combined with electrospray ionization tandem mass spectrometry

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Abstract

Phthalates, bisphenol-A (BPA) and parabens (PBs), organic chemicals widely used in everyday products, are considered to be endocrine disruptors. We propose a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the determination of seven phthalate metabolites, six PBs and BPA in human urine. All three categories of the above endocrine disruptors were simultaneously extracted, from 1 mL of human urine using solid phase extraction. In addition, with a conventional reversed phase LC column, we achieved for the first time the separation of three pairs of structural isomers, namely, iso-/n- butyl paraben, propyl paraben and monobutyl phthalate. LC-MS/MS was operated and tested in both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). ESI was selected for the analysis due to its superior stability and repeatability. The method limit of detection (mLOD), achieved for a single set of HPLC conditions, ranged from 0.01 to 0.84 ng/mL for phthalate metabolites, from 0.06 to 0.24 ng/mL for PBs, and was 2.01 ng/mL for BPA. Derivatisation of BPA with dansyl chloride lowered its mLOD to 0.007 ng/mL. Blank contamination was non-detectable. The present method was successfully applied for the analysis of the above-mentioned compounds in 80 male human urine samples.

Introduction

Endocrine disruptors (EDs) are a group of organic compounds, which cause serious alterations to the normal hormone function in humans and wildlife [1]. EDs interfere with hormone biosynthesis, metabolism or action resulting in a deviation from normal homeostatic control or reproduction in humans [2]. They disrupt the endocrine system by competing with naturally occurring hormones, such as estradiol, or by altering the synthesis and metabolism of these hormones [3]; in addition, there is evidence of reproductive toxicity in laboratory animals and possible health effects in humans [4]. Bisphenol-A (BPA), parabens (PBs) and phthalates are well recognized EDs. Six (6) billion pounds of BPA are produced each year worldwide and over 220,000 pounds of this compound are released yearly into the atmosphere [5]. Phthalates, with over 18 billion pounds used each year, represent one of the world's high production chemical families [6] and PBs, which are used in over 13.200 formulations in nearly all type of cosmetics [7]. Human exposure to these chemicals is occurring through the environment, food intake and the use of products containing them, through inhalation, dermal contact and ingestion [8-12].

Phthalate esters (1,2-diesters) have a variety of common uses. High molecular weight (HMW) phthalates are used in plastic as softeners and low molecular weight (LMW) phthalates are used in personal care products and pharmaceuticals [13-15]. Previous animal tests and epidemiological studies have associated exposure to phthalates with detrimental effects to reproductive and developmental health, as well as increased risk to cancer [8-10]. Phthalates normally follow a metabolic pathway in at least two steps, a hydrolysis (phase-I) where the phthalate diester is hydrolysed into the primary metabolite monoester phthalate and is followed (phase-II) by a conjugation in order to form the more hydrophilic glucuronidated metabolite [16].

The 2,2-bis (4-hydroxyphenyl) propane or bisphenol-A (BPA) is used in industry for the production of many pesticides, resins and polycarbonate plastic. BPA can be found in food and beverage processing, and in many products like dental sealants, personal care products, baby bottles, building materials, flame retardant materials, and optical lenses, materials for the protection of window glazing, DVDs, and household electronics [4,17,18]. Human exposure to BPA is linked to heart diseases, diabetes, liver abnormalities, reproduction adverse effects and alterations in the thyroid [19,20]. BPA is excreted mainly via urine in its free form or in its more hydrophilic glucuronide/sulphate conjugate form [4,21].

PBs is a group of alkyl esters of p-hydroxybenzoic acid. They have low cost of production and demonstrate high chemical stability, inertness, and low acute toxicity [1]. These characteristics made them desirable in

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industry, as antimicrobial preservatives against mould and yeast, in cosmetics, in pharmaceuticals and in food and beverage processing [7]. PBs occur also naturally in food, wine, and plants [22]. *In vitro* studies indicate that PBs induce the growth of MCF-7 human breast cancer cells and influence the expression of estrogen dependent genes [23,24]. In general, PBs are partially hydrolysed by esterases to p-hydroxy-benzoic acid and produce glycine/glucuronide/sulphate conjugates, with increased water solubility that are more amenable to urinary excretion than are the free species [22,25].

In order to assess the exposure of humans, to phthalates, PBs and BPA, measurement of the urinary concentration of free species and their conjugates is essential [26-29]. Several methods suitable for measuring phthalate metabolites, BPA or PBs have been published, but none of them measure all three compound classes [30-35]. Furthermore, only Ultra Performance Liquid Chromatography (UPLC) has been reported to sufficiently separate the structural isomers of propyl- and butyl- paraben [30].

We aimed to develop a suitable chromatographic method for assessing human exposure to the above-mentioned important EDs. Our main goals were, I) to develop a common clean-up procedure for phthalate metabolites, PBs and BPA, present in human urine samples; II) to separate the structural isomers of propyl-paraben, butyl-paraben and monobutyl phthalate metabolite, by using conventional HPLC, instead of UPLC columns and pumps; and III) to succeed the lowest possible detection limits for all the above-mentioned EDs. We thus achieved high sensitivity, selectivity and the capability to analyse large numbers of samples in reasonable times, making the method eminently suitable for epidemiological studies. In order to test the applicability and appropriateness of the present method we applied it for the analysis of phthalate metabolites, BPA and PBs in a large number of male urine samples.

Materials and methods

Analytical standards, reagents and consumables

Mono-ethyl phthalate (mEP), $^{13}C_4$-labeled mEP, mono-n-butyl phthalate (mnBP), $^{13}C_4$-labeled mnBP, mono-iso-butyl phthalate (miBP), mono-benzyl phthalate (mBzP), $^{13}C_4$-labeled mBzP, mono-2-ethyl-hexyl phthalate (mEHP), mono-2-ethyl-5-hydroxy-hexyl phthalate (mEHHP), $^{13}C_4$-labeled mEHHP, mono-2-ethyl-5-oxo-hexyl phthalate (mEOHP), $^{13}C_4$-labeled mNP, 4-methylumbelliferrone, $^{13}C_4$-labeled 4-methylumbelliferrone and D_{16}-Bisphenol-A (D_{16}-BPA) were obtained from Cambridge Isotope Laboratories (USA). 4-methylumbelliferryl

glucuronide, [13]C$_6$-labeled MPB, [13]C$_6$-labeled EPB, [13]C$_6$-labeled n-PPB, [13]C$_6$-labeled n-BPB, dansyl chloride, formic acid (for MS, 98%), solvents (Chromasolv grade for HPLC acetonitrile, ethyl acetate, acetone and methanol) and ammonium hydroxide (28% w/v in water) were purchased from Sigma Aldrich (Germany). Methyl-paraben (MPB), ethyl-paraben (EPB), n-propyl paraben (n-PPB), n-butyl paraben (nBPB), (iso-BPB) iso-butyl-paraben, Bisphenol-A (BPA) and iso-propyl paraben (iso-PPB) were purchased from AccuStandard (USA). Glacial acetic acid was purchased from Carlo Erba (Italy) and orthophosphoric acid (85% w/v in aqueous solution) from Riedel de Haen (Switzerland). Ammonium acetate and monosodium phosphate (reagent grade) were provided by Fluka (Germany). *Escherichia Coli* (*E.Coli*) β-glucuronidase (140 U/mL) was purchased from Roche (Germany). SPE cartridges (Nexus, 60mg sorbent / 3 mL reservoir and 200mg sorbent / 6mL reservoir) were acquired from Varian (USA). High purity water (18.2 MΩ x cm electrical resistivity), was produced by PURELAB Ultra Ionic purification system (ELGA, USA).

Preparation of standards

All standard solutions were stored sealed at -20 °C in Teflon-capped bottles. Phthalate metabolites and 4-methylumbelliferrone standards (native and labelled) obtained in solutions (100 µg/mL in methyl-tert-butyl ether or acetonitrile). 4-methylumbelliferryl glucuronide standard stock solution was prepared in water at 1000 µg/mL. Dansyl chloride standard solution was prepared in acetone at 12.5 mg/mL. PBs, BPA and D$_{16}$-BPA stock solutions were prepared in methanol at 250 µg/mL. Working solutions were prepared at concentrations of 1 µg/mL in 1:1 methanol/water for mass spectrometer optimisation and at 2-8 µg/mL in synthetic urine [31] for spiking samples and calibration curves. Quantitative analysis was based on peak area measurements as ratios with the peak area of their corresponding internal standard. For phthalate metabolites isotopically labelled analogues of native compounds were used as internal standards, except for miBP, mEOHP and mEHP where [13]C$_4$-mnBP and [13]C$_4$-mEHHP were used because the labelled analogues were not commercially available to us for the period of the study. For PBs analysis [13]C$_6$-analogues of methyl-, ethyl, n-propyl and n-butyl parabens were used as internal standards. For BPA analysis, D$_{16}$-BPA was used as the internal standard. Calibration curve solutions, blank, recovery and quality control (QC) samples were prepared in synthetic urine.

Instrumentation

All analyses wereperformed on an LC-MS/MS system consisting of an RP-HPLC chromatograph coupled to a mass spectrometer. Sample injections were performed via a Surveyor Autosampler (Thermo Finnigan, USA).

The chromatographic separation of PBs-BPA-phthalate metabolites was achieved using a Surveyor LC system (Thermo Finnigan, USA), equipped with a BetaSil Phenyl (3 μm, 100 mm x 2.1 mm) analytical column from Thermo Scientific (USA).

Dansylated-BPA/D_{16}-BPA were analysed with a PerfectSil C_8 (3 μm, 125 mm x 2.1 mm, MZ-Analytical, Germany) analytical column. The mass detection was achieved with a TSQ Quantum triple quadrupole mass spectrometer equipped with both ESI and APCI source (Thermo Finnigan, San Jose, USA). The system was controlled by the Xcalibur software, which also was used for the data acquisition, analysis and quantitation.

Mass spectrometry conditions

The mass spectrometer was operated in the selected reaction monitoring (SRM) mode. Source collision induced dissociation (Source CID) and tube lens voltage were set at optimum values for each SRM. Collision gas was Ar at 2.0 mTorr.

For PBs-BPA-phthalate metabolites, ESI in negative mode was chosen as the ionization source. Sheath gas pressure (32-45 arbitrary units, au) and auxiliary gas pressure (20 au) were N_2 and as with spray voltage (4000-4400 V) and Lens 0 offset (1.0-1.3 V), optimum values were set at each time segment. Ion transfer capillary temperature was set at 330°C.

The monitored SRMs of the studied compounds and their isotopically labelled internal standards are presented in Table 1. Dwell time was set at 0.1-0.2 sec except for BPA in which case it was set at 1.5 sec.

Dansylated-BPA was measured with ESI in the positive mode. Sheath gas pressure (35 au) and auxiliary gas pressure (20 au) were N_2. Spray voltage was set at 4000 V and Lens 0 offset at 0.4 V. Ion transfer capillary temperature was set at 300°C. The SRMs are depicted in Table 1. Dwell time was set at 0.75 sec for quantitation ion, 0.55 sec for confirmation ion and 0.2 sec for D_{16}-BPA ion.

HPLC conditions

Injection volume was 20 μL and autosampler settings were as follows: flush volume 1600 μL, wash volume 1600 μL, flush speed 100 μL/s and as wash/flush solvent was used methanol-water 1:1. For PBs-BPA-phthalate metabolites, we modified the gradient used by Silva et al. [32] as depicted in Supplementary Table

S1. Flow rate was set at 350 µL/min. For dansylated-BPA analysis, the applied gradient (200 µL/min flow rate and 0.1% formic acid as mobile phase additive) is presented in Supplementary Table S2.

Sample preparation

After collection, samples were stored at -18 °C and were thawed overnight at 4 °C before analysis. Treatment and clean-up of the samples was based on previous work [33] but modified as follows: Urine samples (1 mL) were transferred to a Falcon tube (polypropylene, 15 mL) and spiked with 100 ng $^{13}C_4$-labeled phthalate metabolites and $^{13}C_4$-labeled 4-methylumbelliferrone, 20 ng $^{13}C_6$-labeled PBs and 200 ng 4-methylumbelliferryl glucuronide. The hydrolysis step, with use of *E.Coli* or *H.Pomatia* β-glucuronidase, has been reported and evaluated in numerous publications [34,36,37]. We have used the *E.Coli* β-glucuronidase hydrolysis as follows: *E.Coli* β-glucuronidase buffer (prepared daily, per sample: 10 µL *E.Coli* β-glucuronidase and 250 µL ammonium acetate buffer, 1M in aqueous solution, pH 6.5) was added to the urine samples and hydrolysis was completed at 37 °C for 90 min. After enzymatic hydrolysis completion, 1 mL of ammonium hydroxide buffer (0.15 % w/v NH_4OH in 1:1 acetonitrile-water) was added to the samples, which were loaded onto the 60 mg solid phase extraction cartridge. The eluents of the first cartridge (60 mg) were acidified with 3 mL monosodium phosphate buffer (0.14 M NaH_2PO_4, aqueous solution, at pH 2) and loaded onto the second solid phase extraction cartridge (200 mg). The eluents from the 200 mg cartridge were discarded. Both cartridges were eluted with 3 mL acetonitrile and 3 mL ethyl acetate each. The eluents of both cartridges (12 mL in total) were combined and evaporated to dryness with a rotational vacuum concentrator RVC 2-25 (Martin Christ, Germany) (60 °C, 20-45 mbar, 150 min for 18 samples). The residues were dissolved in 0.4 mL of water and transferred to a 2 mL autosampler glass vial with a 0.4 mL volume insert. After phthalate metabolites-PBs-BPA LC-MS analysis, in order to enhance BPA detection limit, 160 µL 7% v/v aquatic ammonium hydroxide and 40 µL dansyl chloride 12.5 mg/mL in acetone were added to the autosampler vials containing the samples (200 µL, the rest was discarded) and with 0.5h heating at 65 °C, dansylation was completed and samples were re-analysed with LC-MS. Dansylated-BPA structure is presented in Figure 1. In order to normalise the variability in urine density, an aliquot of 0.5 mL for each urine sample was analysed to determine the creatinine by concentration using the OLYMPUS 2700 immunoassay system (Beckman Coulter, USA).

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Analytical performance

The following parameters were evaluated for the analytical performance of the method: isotopic purity of labelled compounds, recovery and blank levels, method limit of detection/quantitation (mLOD/mLOQ), method limit of detection/quantitation (mLOD/mLOQ), linearity, accuracy and repeatability. Matrix effects cannot be calculated accurately due to the variability of urine density among samples. For this reason, an average matrix effect influence was taken into account for the analyses, by using synthetic urine in calibration curves, blanks, recovery and quality control samples. In order to check the isotopic purity of labelled compounds, we analysed an aqueous solution (200 ng/mL) of each standard, three times. To determine the method recovery, 1 mL of synthetic urine (spiked with native analytes: 1, 5 ng and 50 ng for PBs / BPA and 5, 50 and 100 ng for phthalate metabolites) was analysed three times for each level and internal standards were added before HPLC-MS analysis. To determine possible contaminations during analysis (blank levels), 1 mL of synthetic urine was analysed three times. Instrument limits of detection (iLOD) and quantitation (iLOQ) were set at signal to noise (S/N) ratios equal to 3 and 10 respectively. iLOD and iLOQ were calculated using a calibration curve in synthetic urine in order to take into account signal suppression due to matrix effect. The mLOD and mLOQ were calculated by adjusting iLOD and iLOQ respectively, with the method recovery value and sample condensation factor using the following equations: [mLOD] = [iLOD] / [sample condensation factor * recovery] & [mLOQ] = [iLOQ] / [sample condensation factor * recovery]. The linearity of the method (R^2) was calculated by using the linear equation of calibration curve for each analyte. In order to evaluate the repeatability of the method, a pooled urine sample, spiked with native analytes (1, 5 and 50ng for PBs/ BPA and 5, 50 and 100 ng for phthalate metabolites) was aliquoted and analysed five times for each level. The accuracy of the method was calculated by analysing quality control QC samples (N=5 for each level), which were prepared by spiking the same amounts of native compounds as in repeatability test to synthetic urine.

 Application to real samples

Eighty (80) urine samples were collected from men, working in the city of Heraklion (Island of Crete, Greece). The volunteer samples differed into groups based on their professional activity, namely chemists/biologists working in laboratory (N=28); professors of Chemistry and administration personnel (N=18); bank clerks (N=9); and hairdressers (N=12); miscellaneous others (N=13). It has to be mentioned that the above samples were collected in the frame of the EU funded ENVIROGENOMARKERS project (FP7-ENV-2008-1, Grant Agreement No. 226756), whose goal was to determine only phthalate metabolites. Thus, *E.Coli* β-

glucuronidase was chosen as the hydrolysis enzyme. The samples were initially analysed in order to measure phthalate metabolites and PBs. One injection was performed per sample and the mobile phase contained 0.1% acetic acid. Then, the samples were treated with dansyl chloride in order to enhance BPA detection limits and were re-analysed with LC-MS (one injection / sample). In every set (N=30) of urine samples, one blank and two quality control (QC) samples were analysed. Samples which exceeded calibration range were diluted with synthetic urine and extracted/analysed again.

Results and Discussion

Optimization of mass spectrometry

ESI is the widely used ionisation technique due to its enhanced sensitivity, the low flow rates it requires and its capability to ionise a wide range of analytes [31,34,37,38]. Although, sensitivity was at same levels with APCI, the need for frequent maintenance of this ionisation source (cleaning/replacing corona needles, replacing sample tube) and the significantly larger solvent consumption led us to use ESI. A preliminary optimization of the mass spectrometer parameters took place with direct infusion of each compound at 10 µL/min flow rate via a syringe pump. Optimization was repeated, after HPLC method development, and each compound was re-optimized using the chromatographic conditions (flow rate and solvent type), existing at its retention time. Mobile phase flow (via HPLC pump) and analyte solution flow (via syringe pump) were connected with a T-junction and were driven to the mass spectrometer. Despite the automatic optimization capability of TSQ Quantum, in order to achieve more accurate results, we performed this step manually. In order to achieve appropriate detection limits for phthalate metabolites, PBs and BPA with our mass spectrometer (TSQ Quantum, model acquired in 2003), we chose to follow one SRM per analyte. Furthermore, we have tested and optimized two SRMs per analyte without observing co-eluting peaks in any sample. For dansylated-BPA, two SRMs were monitored.

Optimization of HPLC

For PBs-BPA-phthalate metabolites and with ESI as ionisation source, in addition to the selected BetaSil Phenyl column, we also tested a PerfectSil 120 Phenyl (3 µm, 100 mm x 2.1 mm; MZ-Analytical, Germany) and a Gemini C_{18} (3 µm, 100 mm × 2 mm; Phenomenex, USA) HPLC columns, which did not provide adequate separation and peak shapes. Methanol was tested as the mobile phase; although for PBs and BPA analysis

the results were similar to those obtained using ACN, for phthalate metabolites the separation and peak shapes were unsatisfactory. With APCI, a pair of tandemly connected Hypersil ODS (5 μm, 250 mm × 4.6 mm, MZ-Analytical, Germany) HPLC columns were tested, with both ACN and methanol used as mobile phases. Although separation for PBs was similar to that achieved using a BetaSil Phenyl column with ESI, the gradient was longer, the system pressure and flow rate were significantly higher and the separation of phthalate metabolites was inadequate. The optimum results for the baseline separation of the structural isomers of BPB, PPB and mBP, were accomplished with the BetaSil Phenyl HPLC column. The modified gradient (of a previously reported method [32]; see "Material and methods - HPLC conditions") we used, completed the separation of paraben stuctural isomers. The addition of 0.1% acetic acid to the mobile phase was essential for the retention of phthalate metabolites and their proper separation [34,37,38]. Besides, the acidic mobile phase suppresses the analyte signal, in the negative ESI mode and increases the iLOD for all studied analytes (Table 2) and particularly for BPA [34,37,38]. A chromatogram of a pooled urine sample, spiked with 100 ng of all analytes, is shown in Figure 2.

For dansylated-BPA analysis, between PerfectSil C_8, BetaSil Phenyl and Gemini C_{18} columns, the first demonstrated the best chromatographic performance (peak shape, S/N, matrix components separation). The new gradient, we developed, is presented in Supplementary Table S2, and the chromatographic result of its use for the analysis of a real urine sample is depicted in Figure 3.

In order to increase reproducibility and zero carry-over effects in HPLC, a mixture of 1:1 methanol-water was chosen as syringe cleaning solvent and syringe washes were modified as described in the "Material and methods - HPLC conditions" section. To the best of our knowledge, there is only one report using a UPLC column for the separation of both paraben structural isomers (butyl- and propyl-) [30]. Another study [39], applied an unpublished method, which separates both propyl- and butyl- although only few details are given. In the present study, we achieved for first time, to separate these three isomers using a conventional HPLC column and pump.

Optimization of sample preparation

Applying the modified clean-up procedure for phthalate metabolites (see "Materials and methods-sample separation"), PBs and BPA were also effectively retained (Table 3). Furthermore, to analyse more samples simultaneously and minimize manual intervention, evaporation to dryness was performed with the rotational vacuum concentrator. Derivatisation of phenolic hydroxyl groups with dansyl chloride is a well-known reaction

in organic chemistry and it has applied to enhance mass spectrometric sensitivity of urinary BPA [40,41]. We used aqueous NH$_4$OH to buffer the samples, instead of a non-volatile buffer [40,41] in order to prevent blocking of ion transfer tube after a few injections. Basic pH does not affect BPA deprotonation at ESI source and therefore does not suppress its signal since it elutes at 15.1 min (NH$_4$OH is not retained by the column) and the eluent contains 0.1% formic acid. Dansyl chloride added in excess (0.5 mg per sample) to ensure dansylation for any urine sample (variable concentrations of compounds with phenolic hydroxyls). To the best of our knowledge, this is the first common clean-up protocol reported for the analysis of phthalate metabolites, PBs and BPA.

Analytical performance

Recoveries were higher than 59.1% for all studied metabolites and spiking levels except mEHP (41.5 - 43.5%), which was not eluted effectively from the SPE cartridges possibly due to its high lipophilicity (Table 3). We consequently achieved method limits of detection at the pg/mL - low ng/mL range. Blank contamination was not detectable. The linearity, for the expected concentration range (as presented in Table 3), was excellent (R^2 > 0.99). All isotopically labelled standards were found without detectable contaminations of native compounds. Matrix effects have been reported, for both APCI and ESI analyses, of the studied compounds [33,34,37]. In order to control matrix effects and to perform an accurate analysis, we have used isotopically labelled internal standards for most of the target compounds. Due to their limited commercial availability in the period of study, we used nine labelled internal standards for fourteen target compounds. Repeatability experiments showed standard deviations (STD) <15.0% and accuracy <20.8% for all studied metabolites and spiking levels (Table 3). The chromatogram of a spiked pooled urine sample, analysed for phthalate metabolites-PBs-BPA, is shown in Figure 2 and a urine sample, processed with dansyl chloride, is presented in Figure 3.

Application to real samples

We have applied our method to analyse free and glucuronated metabolites of phthalates, PBs and BPA in eighty (80) urine samples of adult male subjects (see section "Materials and methods-Application to real samples"). Enzymic hydrolysis completion was confirmed both by the absence of 4-methylumbelliferryl glucuronide and by the presence of 4-methylumbelliferrone in the processed samples. Reaction was successful in all analysed samples. In order to assure the determination of the above-mentioned metabolites, we performed the instrumental analysis of the samples twice: I) with acetic acid addition to the mobile phase

to determine phthalate metabolites and PBs II) and with dansylation of the samples in order to obtain the optimal sensitivity for BPA. It has to be underlined that with newer MS instruments significantly lower limits of detection can be achieved and dansylation might be avoided. Chen et al. [37] with an AB-SCIEX API 4000 LC–MS/MS modern instrument (use of 0.1% acetic acid in acetonitrile/H_2O as mobile phase and analysis of 0.2 mL volume sample) obtained mLOD for BPA in the order of 0.3 ng/mL. Urinary concentration data, with the detectability of analytes, are shown in Table 4. Phthalate metabolites were detected, almost in all samples, at significantly higher levels compared to BPA and PBs. Median values of creatinine-adjusted concentrations are shown in Table 5. We attempted to trace differentiations between the professional group exposures. The most interesting outcome is the particularly higher concentrations determined in the hairdressers group samples compared to the other groups, for mEP, MPB and EPB. This observation is possibly justified by the extensive daily use of cosmetic products by these professionals [7,8].

Conclusions

We have developed the first common clean-up procedure for the determination of phthalate metabolites, PBs and BPA. In addition, we elaborated an HPLC-ESI-MS/MS method, with chromatographic characteristics, suitable for the identification and quantification of seven phthalate metabolites, six PBs and BPA in human urine. This method provided a clear separation of n-/iso- structural isomers of butyl-paraben and propyl-paraben. To the best of our knowledge this is reported for the first time by using conventional HPLC columns. The described clean-up procedure and LC/MS method were successfully applied to human urine analysis allowing the determination of the reported analytes in a large number of samples.

Conflict of interest

The authors declare that they have no conflicts of interest.

Compliance with ethical standards

All participants were provided written, informed consent for themselves after having received a complete description of the study, which was approved by the Ethics Committee of the University Hospital in Heraklion, Greece.

Table 1. Selected reaction monitored for phthalate metabolites, parabens, BPA, dansylated-BPA and their isotopically labelled analogues

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
MPB	151.1	92.1	14
$^{13}C_6$-MPB	157.1	98.1	14
EPB	165.1	92.1	30
$^{13}C_6$-EPB	171.1	98.1	30
nPPB / iso-PPB	179.1	92.1	40
$^{13}C_6$-nPPB	185.1	98.1	40
nBPB / iso-BPB	193.1	92.1	33
$^{13}C_6$-nBPB	199.1	98.1	30
mEP	193.1	77.1	10
$^{13}C_4$-mEP	197.1	79.1	10
mnBP / miBP	221.1	77.1	10
$^{13}C_4$-mnBP	225.1	79.1	10
mEHHP	293.2	121.1	10
$^{13}C_4$-mEHHP	297.2	124.1	10
mEOHP	291.2	121.1	10
mBzP	255.2	105.1	10
$^{13}C_4$-mBzP	259.2	107.1	10
mEHP	277.2	134.1	10
BPA	227.2	212.2	18
D16-BPA	241.2	223.2	19
4methyl-umbelliferrone	177.1	133.1	26
$^{13}C_4$-4methyl-umbelliferrone	179.1	135.1695.5	26
Dansylated BPA quantitation SRM	695.5	171.1	40
Dansylated BPA confirmation SRM	695.5	235.1	38
Dansylated D_{16}-BPA	709.5	170.1	35

Table 2. Comparison of instrument / method Limits of Detection / Quantification for different conditions

| Analyte | iLOD-iLOQ (ng/mL) | | | mLOD-mLOQ (ng/mL) | | |
	iLOD-iLOQ with 0.1% acetic acid (ng/mL)	iLOD-iLOQ without acetic acid (ng/mL)	iLOD-iLOQ with dansylation (ng/mL)	with acetic acid in mobile phase	without acetic acid in mobile phase	with dansylation
mEP	-	-	-	0.40-1.33	-	-
mnBP	-	-	-	0.25-0.83	-	-
miBP	-	-	-	0.41-1.37	-	-
mBzP	-	-	-	0.02-0.07	-	-
mEHP	-	-	-	0.84-2.80	-	-
mEHHP	-	-	-	0.01-0.03	-	-
mEOHP	-	-	-	0.18-0.60	-	-
MPB	0.28-0.93	0.14-0.47	-	0.12-0.40	0.06-0.20	-
EPB	0.13-0.43	0.12-0.40	-	0.06-0.20	0.06-0.18	-
iso-PPB	0.41-1.37	0.23-0.77	-	0.24-0.80	0.13-0.45	-
nPPB	0.33 -1.10	0.19-0.63	-	0.15-0.50	0.09-0.29	-
iso-BPB	0.15-0.50	0.07-0.23	-	0.08-0.27	0.04-0.13	-
nBPB	0.15-0.50	0.08-0.27	-	0.07-0.23	0.04-0.12	-
BPA	4.43-14.76	0.16-0.53	0.008-0.026	2.01-6.69	0.07-0.24	0.007-0.024

Table 3. Analytical performance characteristics

Compound	Linearity range (R^2>0.99) (ng/mL)	Recovery % (n=3, ± SD[a])			Accuracy %			Repeatability %		
		Low	Medium	High	Low	Medium	High	Low	Medium	High
mEP	0.5-512	60.5±6.4	69.5±4.5	68.5±7.5	9.2	5.5	7.3	2.6	2.6	5.2
mnBP	0.5-512	80.7±7.8	87.2±7.8	70.6±8.2	5.7	6.6	4.2	2.0	7.0	3.5
miBP	0.5-512	73.9±9.6	76.7±5.4	67.3±7.4	9.0	3.9	3.1	6.2	2.7	4.2
mBzP	0.5-512	64.8±5.4	69.4±6.6	67.0±6.7	7.4	4.0	3.8	6.8	5.5	3.5
mEHP	1-512	43.5±5.2	40.8±1.8	41.5±16.0	20.8	19.3	5.2	7.9	2.8	5.7
mEHHP	0.5-512	64.2±7.8	62.1±1.8	66.6±7.5	9.1	7.6	6.3	5.2	5.7	5.6
mEOHP	0.5-512	59.1±5.1	79.6±7.9	68.3±7.1	15.6	8.5	7.0	6.9	7.6	6.2
MPB	0.25-512	95.0±8.6	99.8±4.3	91±3.4	19.5	17.8	10.1	11.0	2.8	1.2
EPB	0.25-512	99.0±8.2	103.2±2.6	88±6.7	12.7	10.3	13.1	8.0	6.0	1.5
iso-PPB	0.25-512	75.9±11.5	70.7±6.1	69±8.0	19.9	0.1	3.9	7.0	4.7	1.9
nPPB	0.25-512	89.8±4.6	95.4±4.3	86±6.9	5.1	3.9	3.7	15.0	1.4	3.4
iso-BPB	0.25-512	75.3±5.1	80.8±3.8	77±5.7	8.6	6.5	7.5	9.3	1.9	1.8
nBPB	0.25-512	69.8±4.8	87.7±2.7	83±5.6	15.2	1.1	1.1	8.6	1.8	1.5
BPA (dansylated)	0.05-64	75.2±4.2	78.9±4.3	88±5.6	3.1	1.9	1.4	2.4	2.6	1.6

[a]SD: standard deviation

Table 4. Concentration values in ng/mL of urine for 80 urine samples

	Median (ng/mL)	Maximum (ng/mL)	Average (ng/mL)	Detectability %
MPB	5.5	3868.6	76.7	100
EPB	0.4	205.4	5.9	94
iso-PPB	N.D.[b]	0.2	N.D.	1
nPPB	0.2	806.6	21.6	60
iso-BPB	N.D.	0.9	N.D.	13
nBPB	N.D.	163.0	2.2	15
mEP	100.3	3649.8	218.4	100
miBP	45.2	352.1	58.1	100
mBP	29.9	144.8	41.8	100
mEHHP	52.1	243.0	69.2	100
mEOHP	34.6	122.4	40.8	99
mBzP	11.3	205.3	15.6	100
mEHP	15.0	99.2	20.5	91
BPA	0.6	3.93	0.85	96

[b]N.D.: Not Detected

Table 5. Median concentration values in µg/g of urinary creatinine for 80 samples

	MPB	EPB	iso-PPB	nPPB	nso-BPB	nBPB	BPA	mEP	miBP	mBP	mEHHP	mEOHP	mBzP	mEHP
Chemists /Biologists	2.0	0.2	N.D.[c]	0.1	N.D.	N.D.	0.4	62.5	26.1	20.7	41.9	27.8	9.2	10.9
Professors / Administrative personnel	2.9	0.3	N.D.	N.D.	N.D.	N.D.	0.4	42.0	30.6	19.9	27.2	15.0	5.2	5.8
Bank Clerks	3.5	0.3	N.D.	N.D.	N.D.	N.D.	0.3	64.4	40.9	27.3	22.2	15.4	4.7	10.6
Hairdressers	10.5	1.3	N.D.	0.5	N.D.	N.D.	0.3	146.8	29.4	20.2	27.4	15.7	9.0	13.4
Various	1.6	0.2	N.D.	N.D.	N.D.	N.D.	0.3	46.2	22.8	12.7	26.0	15.5	5.4	6.4

[c]N.D.: Not detected

Figures
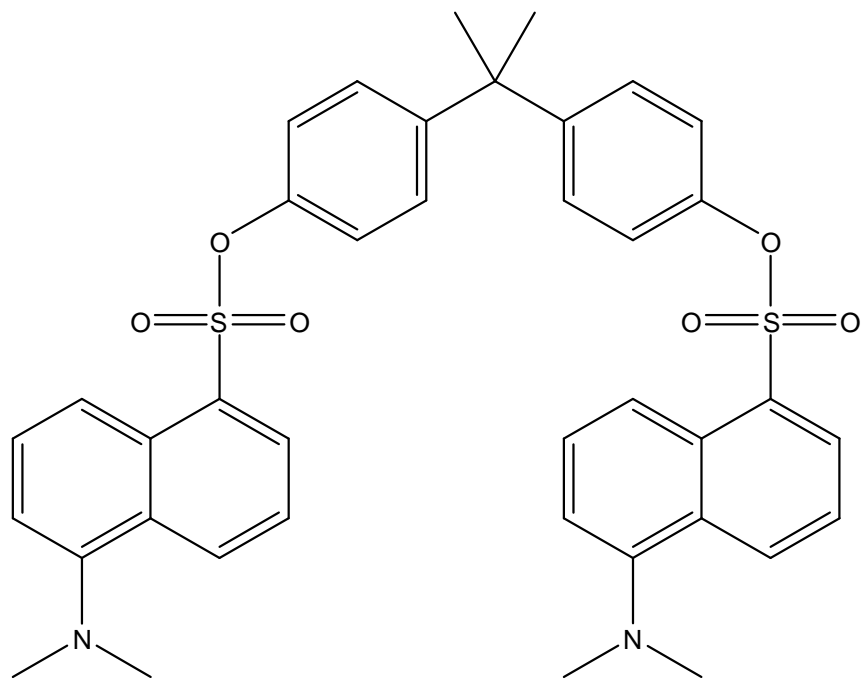
Fig.1 Dansylated BPA structure
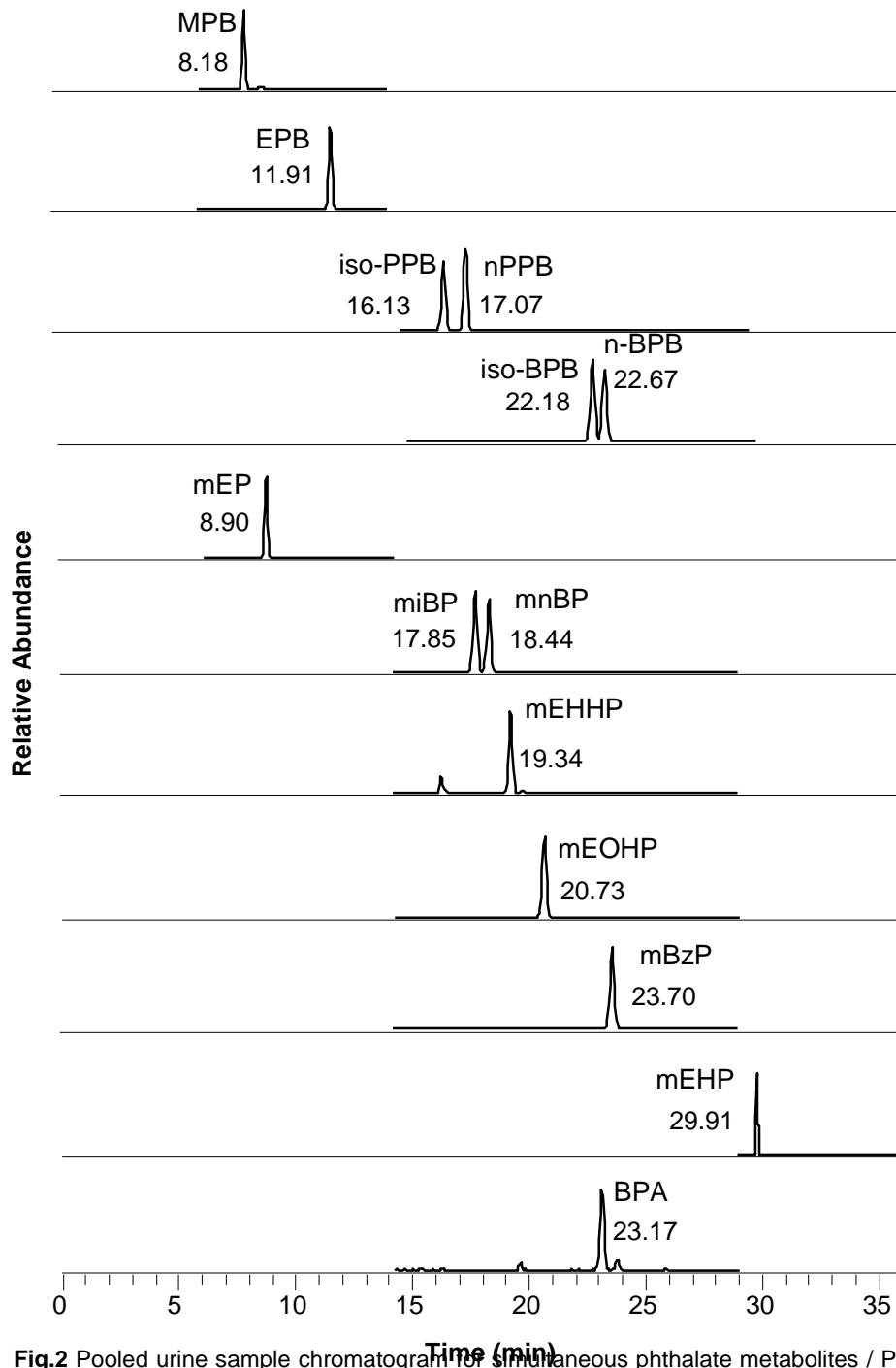
Fig.2 Pooled urine sample chromatogram for simultaneous phthalate metabolites / PBs / BPA analysis and their SRMs, peak intensities and retention times in minutes
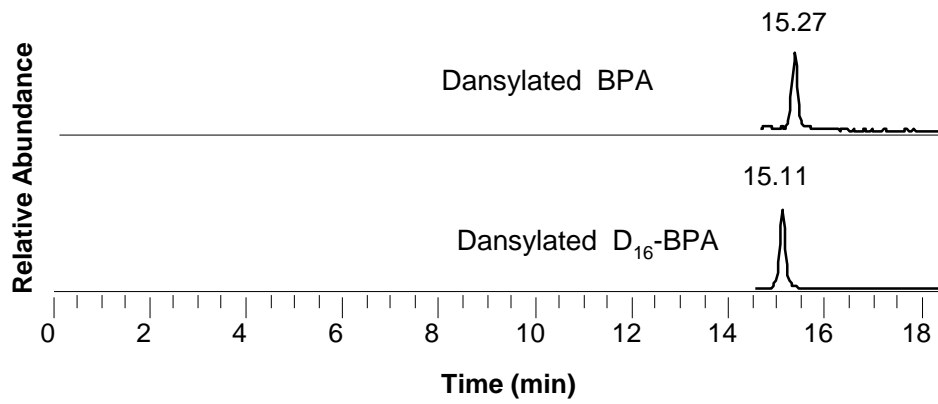
Fig.3 Urine sample chromatogram for dansylated BPA / D_{16}-BPA and their SRMs, peak intensities and retention times in minutes

Supplementary Material

Authors: Antonis Myridakis, Eirini Balaska, Christina Gkaitatzi, Antonis Kouvarakis and Euripides G. Stephanou

Title: Determination and separation of bisphenol A, phthalate metabolites and structural isomers of parabens in human urine with conventional high-pressure liquid chromatography combined with electrospray ionization tandem mass spectrometry

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Table S2. Gradient program for phthalate metabolites paraben and BPA analysis

Time (min)	(0.1 % acetic acid in acetonitrile) %	(0.1 % acetic acid in water) %
0	4	96
0.1	4	96
1.0	15	85
14.0	25	75
27.0	35	65
28.0	100	0
32.0	100	0
33.0	4	96
36.0	4	96

Table S2. Gradient program for dansylated-BPA analysis

Time (min)	(0.1 % formic acid in acetonitrile) %	(0.1 % formic acid in water) %
0	60	40
0.1	60	40
15.5	100	0
17.0	100	0
17.1	60	40
18.4	60	40

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Appendix 2

Title: Phthalate esters, parabens and bisphenol-A exposure among mothers and their children in Greece (RHEA cohort)

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Abstract

Exposure to endocrine disruptors, used as additives, preservatives, plasticizers and solvents in numerous consumer products, might cause adverse health effects. Humans exposed to these chemicals, metabolise and excrete them mostly via urine. Urinary metabolite concentrations are used as biomarkers of exposure. We evaluated the exposure to phthalates, parabens and bisphenol-A in 4-month pregnant women and their children at 2 years of age. Concentrations of eight phthalate metabolites, six parabens and bisphenol-A were measured in 239 mother-child pairs of the "Rhea" cohort in Greece. Concentration levels in mother and children were comparable with corresponding concentrations in other countries worldwide. Low two-tailed spearman correlation coefficients (CC 0.1-0.2, p-value <0.01) were observed for di-ethyl phthalate (DEP), di-n-butyl phthalate (DnBP), butyl-benzyl phthalate (BBP) and ethyl paraben (EPB) between mothers and their children. We observed higher median daily intake (DI_u) for mothers (e.g. di-ethyl phthalate 6.9 µg d^{-1} kg^{-1}) than for their children (1.4 µg d^{-1} kg^{-1}) samples for all examined compounds, except for di-2-ethylhexyl phthalate (DEHP) and bisphenol-A. Principal component analysis (PCA) indicated two main sources of exposure (plastic related and personal care-hygiene products) for phthalates, parabens and bisphenol-A. Differences in DEHP metabolism were observed among mothers-children and female-male children.

Highlights:

- Concentration levels of eight phthalate metabolites, six parabens and bisphenol-A

- Two hundred and thirty nine (239) mother child pairs from Rhea Cohort – Greece

- Some same exposure sources at pregnancy and after birth

- Two distinct exposure sources: plastic and personal care products

- Differentiations in DEHP (di-2-ethylhexyl phthalate) metabolism

Keywords: phthalates, bisphenol-A, parabens, mother-child pairs, Rhea cohort

Abbreviations: BPA, bisphenol-A; BBP, butyl-benzyl phthalate; CC, correlation coefficient; C_u, metabolite concentration, µg/L; DEHP, di-2-ethylhexyl phthalate; DEP, di-ethyl phthalate; DiBP, di-iso-butyl phthalate; DI_u, daily intake calculated using urinary metabolites; DnBP, di-n-butyl phthalate; EPB, ethyl paraben; HPLC, high performance liquid chromatography; isoBPB, iso-butyl paraben; isoPPB, iso-propyl paraben; F_{ue}, urinary excretion factor; mBzP, mono-benzyl phthalate; mCOP, mono-carboxy-octyl phthalate; mEHHP, mono-2-ethyl-5-hydroxy-hexyl phthalate; mEOHP, mono-2-ethyl-5-oxo-hexyl phthalate; mEP, mono-ethyl phthalate; mLOD, method limit of detection; mnBP, mono-n-butyl phthalate; mNP, mono-iso-nonyl phthalate; MPB, methyl paraben; MW_1, molecular weight of phthalate diester; MW_2, molecular weight of phthalate metabolite; nBPB, n-butyl-paraben; NC, not calculated; ND, not detected; nPPB, n-propyl paraben; NR, not reported; PB, parabens; PCA, Principal Component Analysis; PhE, phthalate esters; RfD, reference dose; SPE, solid phase extraction; SRM, selected reaction monitoring; RMR, relative metabolic rate; RMR_1, mEHHP/mEHP molar concentrations ratio; RMR_2, mEOHP/mEHHP molar concentrations ratio; TDI, tolerable daily intake; UPLC, ultra performance liquid chromatography; W, body weight

1. Introduction

Endocrine disruptors are a group of organic chemicals, which alternate intensively to the normal hormone function of humans (World Health Organization 2012). They interfere with hormone biosynthesis, metabolism or action resulting in a deviation from normal homeostatic control or reproduction in humans (Diamanti-Kandarakis and others 2009). They disrupt the endocrine system by competing with naturally occurring hormones such as estradiol, or by altering the synthesis and metabolism of these hormones (National Institute of Health 2010). There is evidence of reproductive toxicity in laboratory animals and possible health effects in humans (Crinnion 2010). Pregnant mothers (their embryos) and children are the most vulnerable populations to endocrine disruptor exposure (World Health Organization 2012). Phthalates (PhE), with around one million tons annual production in Europe (AgPU 2006), bisphenol-a (BPA) with about 3.6 million tons annual global production (Geens and others 2012) represent some of the world's highest production chemicals and parabens (PB) which are used in over 13.200 formulations in nearly all type of cosmetics (Elder 1984), are well recognized endocrine disruptors (Witorsch and Thomas 2010). Human exposure to these chemicals is occurring through the environment, food intake and the use of products containing them, through inhalation, dermal contact and ingestion (ATSDR DEHP 2001; ATSDR DEP 1995; ATSDR DnBP 2001; Meeker 2010; Soni and others 2001).

PhE (1,2-diesters of phthalic acid) have a variety of common uses. Higher molecular weight PhE are used in plastic as softeners and lower molecular weight PhE are used in personal care products and pharmaceuticals (Wormuth and others 2006). Previous animal tests and epidemiological studies have associated exposure to PhE with detrimental effects to reproductive and developmental health, as well as increased risk in cancer (ATSDR DEHP 2001; ATSDR DEP 1995; ATSDR DnBP 2001). Once ingested/absorbed, lower molecular weight PhE are hydrolysed to their monoesters, while higher molecular weight PhE can be subsequently oxidized to several other metabolites from their primary monoesters. Primary and secondary metabolites from PhE breakdown can be further biotransformed to their glucoronide analogues before being excreted via urine (Calafat and others 2006).

PB is a group of alkyl esters of p-hydroxybenzoic acid. They have low cost of production, and demonstrate chemical stability, inertness, and low acute toxicity (World Health Organization 2012). These characteristics made them desirable in industry, as antimicrobial preservatives against mould and yeast, in cosmetics, in pharmaceuticals and in food and beverage processing (Elder 1984). PB also occurs naturally in

food, wine, and plants (Soni and others 2005). *In vitro* studies indicate that PB induce the growth of MCF-7 human breast cancer cells and influence the expression of estrogen dependent genes (Byford and others 2002). In general, PB are partially hydrolysed by esterases to p-hydroxy-benzoic acid and produce glycine/glucuronide/sulphate conjugates, with increased water solubility that are more amenable to urinary excretion than are the free species (Soni and others 2005; Wang and James 2006).

BPA (4,4'-(propane-2,2-diyl) diphenol) is widely used in polycarbonate and epoxy resin production. It can be found in many products like dental sealants, food packaging, beverage cans, personal care products, baby bottles, building materials, flame retardant materials, optical lenses, DVDs and household electronics (Geens and others 2012; Staples and others 1998). After epidemiological studies in human beings and experiments in mice, BPA exposure is suspected for causing several adverse health effects, as cancer, obesity and disorders in endocrine, renal and reproductive systems (Rubin 2011). BPA is excreted mainly via urine in its free form or in its more hydrophilic glucuronide/sulphate conjugate form (Chapin and others 2008).

Table 1. Studied endocrine disruptors and their method limits of detection (mLOD)

Parent compounds	Studied metabolites	Method limit of detection (mLOD) ng/mL urine
di-ethyl phthalate (DEP)	mono-ethyl phthalate (mEP)	1.3
di-n-butyl phthalate (DiBP)	mono-n-butyl phthalate (miBP)	2.1
di-iso-butyl phthalate (DnBP)	mono-iso-butyl phthalate (miBP)	2.5
di-2-ethylhexyl phthalate (DEHP)	mono-2-ethylhexyl phthalate (mEHP)	0.8
	mono-2-ethyl-5-hydroxy-hexyl phthalate (mEHHP)	0.9
	mono-2-ethyl-5-oxo-hexyl phthalate (mEOHP)	1.8
butyl-benzyl phthalate (BBP)	mono-benzyl phthalate (mBzP)	2.2
di-iso-nonyl phthalate (DNP)	mono-isononyl phthalate (mNP)	2.2
methyl paraben (MPB)		0.06
ethyl paraben (EPB)		0.06
iso-propyl paraben (isoPPB)		0.13
n-propyl paraben (nPPB)		0.09
iso-butyl paraben (isoBPB)		0.04
n-butyl paraben (nBPB)		0.04
Bisphenol-A (BPA)		0.01

In order to assess the exposure of PhE and PB, in humans, measurement of the urinary concentration of free species and their conjugates is essential (Silva and others 2003a; Ye and others 2006). In this study, 8 PhE metabolites, 6 PB and BPA (Table 1) were measured in 239 mother-child pairs in Heraklion, Crete (Rhea cohort). We aimed: a) to evaluate, for the first time, the levels of exposure to PhE, PB and BPA in

Greece, b) to investigate the potential correlation in the exposure levels between the mothers and their children, c) to estimate the daily intake (DI$_u$) of the PhE, PB and BPA d) to compare our results with other similar studies worldwide and e) to attempt the assessment of potential exposure sources.

2. Materials and methods

2.1 Study population

The present study comprises mothers and children, part of the "Rhea" cohort, from the island of Crete (Greece). The "Rhea" cohort has been presented in detail previously (Chatzi and others 2009; Patelarou and others 2011). Briefly, women who became pregnant during February 2007-February 2008 participated in the study. Women, residents of the study area, >16 years of age, completed face-to-face interviews and provided blood and urine samples, visiting a participating hospital or private clinic during the 10th–13th week of gestation. Participating women were contacted again during the 14th-18th and 28th-32nd weeks of pregnancy and at birth. Children samples (103 females-136 males) were collected during March 2009-June 2011. Spot urine samples were collected at around the fourth month of pregnancy for mothers and at 2.3 ± 0.72 years for children; mean age ± standard deviation). A total of 239 mother-child pairs were monitored.

Urine samples were collected in urine boxes and stored at 4°C until procession. Within 4 hours, samples were aliquoted in 4mL cryovials and stored at -80°C. Urine boxes and cryovials were made of polypropylene and checked for possible contaminations. Creatinine levels were 0.50±0.31 g/L (arithmetic mean ± standard deviation) for children and 1.20±0.67 for mothers. Samples with creatinine values, out of 0.3-3 g/L range for mothers (Barr and others 2005a; Barr and others 2005b) and out of 0.1-3.0 range for children were excluded from analysis. We did not applied the same exclusion criteria for mothers and children, because creatinine values below 0.3 mg/L in children do not necessarily indicate excessive dilution but are indicative of lower muscle mass compared to adults (Koch and others 2011). All participant mothers provided written, informed consent for themselves and their child after having received a complete description of the study, which was approved by the Ethics Committee of the University Hospital in Heraklion, Greece.

2.2 Instrumental analysis

An aliquot of each sample (1 mL) was analysed for eight PhE metabolites and six PB (Table 1) during February 2010-December 2012. Our primary goal was to measure only PhE metabolites for an EU funded FP7 project

(Envirogenomarkers), so we used E.Coli β-glucuronidase for the enzymatic hydrolysis of conjugated endocrine disruptors in urine. As a result, we obtained total PhE metabolites (free and glucuronated) but not all the PB and BPA species (sulfated metabolites demand H.Pomatia β-glucuronidase (Dewalque and others 2014; Volkel and others 2002). Treatment and clean-up of the samples was based on previous work (Silva and others 2003b) but modified in several steps. Urine samples (1 mL) were spiked with internal standards and E.Coli β-glucuronidase buffer (per sample: 10 μL E.Coli β-glucuronidase and 250 μL ammonium acetate buffer, 1M in aqueous solution, pH 6.5) was added. Hydrolysis was completed at 37 °C for 90 min. After enzymatic hydrolysis completion, 1 mL of ammonium hydroxide buffer (0.15 % w/v NH$_4$OH in 1:1 acetonitrile-water) was added to the samples, which were loaded onto the first solid phase extraction cartridge (Varian Nexus, 60 mg). The eluents of the first cartridge were acidified with 3 mL monosodium phosphate buffer (0.14 M NaH$_2$PO$_4$, aqueous solution, at pH 2) and loaded onto the second solid phase extraction cartridge (Varian Nexus, 200 mg). The eluents from the second cartridge were discarded. Both cartridges were eluted with 3 mL acetonitrile and 3 mL ethyl acetate each. The eluents of both cartridges (12 mL in total) were combined and evaporated to dryness with a rotational vacuum concentrator RVC 2-25 (Martin Christ, Germany) (60 oC, 20-45 mbar, 150 min for 18 samples). The residues were dissolved in 0.4 mL of water and transferred to a 2 mL autosampler glass vial with a 0.4 mL volume insert. After PhE metabolites and PB LC-MS analysis, in order to enhance BPA detection limit, 160 μL 7% v/v aquatic ammonium hydroxide and 40 μL dansyl chloride 12.5 mg/mL in acetone were added to the autosampler vials containing the samples (200 μL, the rest was discarded) and with 0.5h heating at 65 °C, dansylation was completed and samples were re-analysed with LC-MS. Two new gradient elution programs were developed a) for PhE metabolites and PB analysis: mobile phase was acetonitrile and water, both containing 0.1% acetic acid; flow rate at 350 μL/min; 36 min run; from 4% organic phase to 100% and back to 4%; Thermo Phenyl Betasil column, 3μm, 100mm x 2.1 mm b) mobile phase was acetonitrile and water, both containing 0.1% formic acid; flow rate at 200 μL/min; 18.5 min run; from 60% organic phase to 100% and back to 60%; MZ PerfectSil C$_8$ (3μm, 125mm x 2.1mm). In order to minimise BPA limits of detection, after PB and PhE metabolites LC-MS analysis, extracted samples were derivatised with dansyl chloride and dansylated-BPA was monitored. Detection performed with a TSQ Quantum triple quadrupole (Thermo Finnigan, San Jose, USA) with ESI source operated in negative mode for PhE metabolites/ PB and in positive mode for dansylated-BPA. Mass spectrometer was set in selected reaction monitoring (SRM).

Method limits of detection of studied compounds are presented in Table 1. Isotopically labelled analogues of studied compounds were used as internal standards. Labelled isoPPB, isoBPB, miBP and mEHP were not commercially available at the time of analysis and labelled nPPB, nBPB, mnBP and mEOHP were used as their internal standards. Blank contamination was lower than detection limits and recovery was > 59% for all studied compounds except mEHP which was at 44%. Standard deviation of accuracy and repeatability tests were <13.1% and <6.2% respectively. Method was linear (R^2>0.99) for the range LOD-512 ng/mL. Samples exceeding the upper limit of linearity were reanalysed, diluted with nanopure water. Two quality controls samples (spiked pooled urine) and two blank samples (synthetic urine) were analysed with every forty six (46) urine samples. A second aliquot of 0.5 mL urine was analysed for creatinine concentration using the OLYMPUS 2700 immunoassay system (Beckman Coulter, USA). All samples were measured in duplicates. The amount of each sample was quantified by the standard curve performed in each assay.

2.3 Statistical analysis

Statistical analysis was performed with the software SPSS 22.0 (IBM Corporation, U.S.A.). Measurements below mLOD (not detected) were substituted by the mLOD divided by the square root of 2 (two) (Hornung 1990), as the most widely used way to handle non-detects in such type of studies (Ferguson and others 2014; Song and others 2013). Arithmetic mean, minimum, median, 95th percentile, maximum, geometric mean and 95% confidence interval of geometric mean (95 % CI) values were calculated for both unadjusted/creatinine-adjusted concentrations and estimated daily intake data.

The daily intake of PhE and PB estimated by adapting a commonly used toxicokinetic model to our data (Equation 1) (Beko and others 2013; Dirtu and others 2013; Ma and others 2013), where: DI_u (Daily Intake calculated using urinary metabolites, µg×d^{-1}×kg^{-1} of body weight), C_u (metabolite concentration, µg/L), F_{ue} (urinary excretion factor, molar ratio of parent compound intaken to metabolite excreted), MW_1 (molecular weight of phthalate diester, g/mol), MW_2 (molecular weight of PhE metabolite, g/mol) and W (body weight, kg). Especially for PB, ratio (MW_1/MW_2) was set equal to 1 and we included a multiplier (P) of 1.72 (for n-BPB and iso-BPB 1.04) since we measured free plus glucuronated PB which represent 58% of the total urinary PB concentrations (glucuronated n-BPB and iso-BPB, 96%) (Dewalque and others 2014). Since glucuronated and free forms of BPA practically represent the total BPA excretion, we didn't used any normalisation factor for e.g. the sulphated species (Volkel and others 2002).

Equation 1: $DI_u = \frac{C_u \times V_u \times P \times MW_1}{W \times F_{ue} \times MW_2}$ (μg of endocrine disruptor $\times d^{-1} \times kg^{-1}$ of body weight)

F_{ue} values for mEHHP and mEOHP were taken from (Koch and others 2004a) and (Koch and others 2005a), for mBzP and mnBP from (Anderson and others 2001), for PB from (Ma and others 2013) and since for miBP and mEP, F_{ue} values were not available, we used the same with mnBP. F_{ue} value for BPA was set equal to 1 since BPA is excreted via urine nearly 100% during an 24h period (Volkel and others 2002). V_u considered 2 L for mothers (Guo and others 2011) and 0.0224 L/kg body weight for children (Miller and Stapleton 1989; Szabo and Fegyverneki 1995). We chose to use a value for children volume urine, which doesn't take into account body weight when it is applied to our toxicokinetic model because children especially in ages of this study grow rapidly and a stable volume of urine as in used mothers could introduce uncertainty in daily intake estimation.

In order to investigate possible differentiations in DEHP metabolism among population groups, relative metabolic rate (RMR) of DEHP was calculated as described in literature (Boas and others 2010; Song and others 2013). Briefly, RMR_1 (1st step of metabolism) considered as the molar concentration ratio of mEHP/mEHHP and RMR_2 (2nd step) the ratio of mEHHP/mEOHP. Only samples with positive detection in all three DEHP metabolites were used. For two-tailed Spearman correlations, creatinine adjusted molar (μmol/g) concentrations were used. For two-tailed Pearson correlations, unnormalised RMR values were used since RMR data were not skewed.

Principal component analysis (PCA) with Kaiser normalisation and Mann-Whitney U test (concentration levels gender-based comparison, Table 2) were applied to molar, unadjusted for creatinine, log10 transformed concentrations. Wilcoxon signed ranked (daily intake mother-child pairs based comparison) test was applied to log10 transformed DI_u data. Independent samples t-test was applied to unnormalised RMR data (mothers-children as independent populations and male-female children comparisons) and to gender-based creatinine levels comparison. For PCA and correlation studies, molar concentration levels were used and mEHHP-mEOHP concentrations summed as DEHP metabolites.

Analytes with detectability lower than 50% (isoPPB, isoBPB and nBPB) were excluded from PCA, correlation studies and geometric mean calculation (Table 3). DEHP-DI_u considered as the arithmetic mean of DI_u for mEHHP and mEOHP. mEHP was excluded from the above analyses (PCA, correlation studies, DI_u) due to its relatively lower levels in urine and shorter half-life compared to the other two measured DEHP

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metabolites, mEHHP and mEOHP (Frederiksen and others 2007; Koch and others 2005a; Silva and others 2006a; Silva and others 2006c; Wittassek and Angerer 2008) (Table 4).

2.4 Comparison with other studies worldwide

Literature search for similar studies was performed via EndNote X7 (Thompson Reuters) in PubMed database on December 04, 2014. The search criteria were the following: for PhE metabolites/BPA, titles containing (*phthalate or bisphenol-a or bpa) and (child* or mother* or pregnan* or women) and for PB titles containing *paraben*. The selection criteria were: for studies measuring total metabolites (free, glucuronated and sulphated) from pregnant women or children for over 200 urine samples for PhE metabolites/BPA and 100 for PB, with creatinine normalised median concentrations available for BPA or at least for 4 common PhE metabolites or 4 common PB with our study.

Initially, 796 articles were identified: 93 hits for (*phthalate* and child*), 10 for (*phthalate* and mother*), 63 for (*phthalate* and pregnan*), 46 for (*phthalate* and women), 44 for (bisphenol-a and pregnan*), 53 for (bisphenol-a and child*), 8 for (bisphenol-a and mother*), 41 for (bisphenol-a and women), 3 for (bpa and women), 1 for (bpa and mother*), 2 for (bpa and pregnan*), 4 for (bpa and child*) and 428 for *paraben*. Fifteen (15) of them fulfilled the search criteria: (Boas and others 2010; Braun and others 2011; Braun and others 2009; Casas and others 2013; Frederiksen and others 2013; Harley and others 2013; Hong and others 2013; Kasper-Sonnenberg and others 2014; Lee and others 2014; Mortamais and others 2012; Quiros-Alcala and others 2013; Tefre de Renzy-Martin and others 2014; Teitelbaum and others 2012; Wang and others 2014; Zeman and others 2013)References of the selected papers were also checked but no additional articles identified. In case of concentrations given separately for male/female children or at different time-points of pregnancy, the arithmetic mean of the given median values was used.

3. Results and discussion

3.1 Exposure levels

3.1.1 Concentration levels

The metabolites mEP, mnBP, miBP, mEOHP, mEHHP, mBzP , MPB, EPB, nPPB and BPA were detected in >90.8% of mother samples and in >86.2% of children samples (239 mother-child pairs), while mEHP was detected in the 72.7 % of mother and 57.3% of children samples; isoPPB, isoBPB and nPPB ranged from 12.0% to 38.6% detectability for mothers and 2.1% to 25.9% for children (Table 3). mNP was not detected in any of the analysed samples, thus we do not consider it as a proper urine biomarker for DiNP exposure. Secondary metabolites, (e.g. mCOP, mono-carboxy-octyl phthalate) of this PhE should be used as biomarkers. The same conclusions concerning mNP were also drawn in other studies (Koch and others 2007; Silva and others 2006b). Due to the variable density of urine spot samples (Barr and others 2005b), we preferred to use creatinine normalisation in order to rank the concentration levels of studied compounds.

Table 2. Gender-based comparison of children arithmetic mean concentration levels (ng/mL)

	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	mEHP	MPB	EPB	isoPPB	nPPB	isoBPB	nBPB	BPA
Male	87.88	67.41	46.29	57.92	37.02	15.08	6.98	223.09	17.99	0.09	30.26	0.04	1.20	4.34
Female	67.79	55.82	48.27	42.24	32.31	10.14	7.06	165.33	27.35	0.20	19.66	0.05	0.71	4.62
p-value	0.006	0.004	0.011	0.005	0.015	0.009	0.606	0.095	0.425	0.807	0.036	0.246	0.093	0.256

Among the creatinine adjusted median levels of PhE metabolites, mEP was the most abundant for mothers (133.6 µg/g) and miBP for children (101.6 µg/g); miBP concentrations were in higher levels compared to mnBP for both categories; from DEHP metabolites, mEHHP and mEOHP were in higher levels compared to mEHP also for both categories; mBzP was the less abundant detected PhE metabolite (Table 3). Concerning creatinine adjusted median PB levels, MPB was the most abundant for both categories (mothers: 121.9 µg/g, children: 42.6 µg/g), but for mothers, nPPB levels were higher compared to EPB and for children EPB levels were higher to nPPB; the other three PB had median values < mLOD (Table 3). BPA levels were found at 1.1 µg/g for mothers and at 5.2 µg/g for children. When children were categorized by gender, males exhibited, statistically significant (Mann-Whitney U test; p-value<0.05), higher unnormalised concentrations (ng/mL) for nPPB and all PhE metabolites except from mEHP (Table 2).

Table 3. Descriptive statistics of PhE metabolites, PB and BPA urinary concentrations

	Detectability %	Minimum	Median	95% percentile	Maximum	Arithmetic mean	Geometric mean	95% CI
239 mothers, ng of analyte/mL urine (µg of analyte / g creatinine)								
mEP	100.0	2.6 (4.8)	133.9 (132.6)	1462.9 (1230.9)	4103.7 (3993.8)	360.9 (323.2)	141.9 (143.5)	119.3-171.8 (122.0-169.8)
miBP	98.0	<LOD[a]	39.2 (38.7)	189.4 (131.9)	616.1 (720.0)	62.0 (54.7)	36.7 (37.1)	32.2-42.3 (33.2-41.6)
mnBP	95.9	<LOD	36.1 (33.2)	210.5 (157.1)	94670.7 (48799.3)	463.9 (260.2)	32.1 (32.5)	27.3-38.5 (28.4-37.5)
mEHHP	96.4	<LOD	25.7 (24.4)	125.5 (107.8)	6267.3 (5095.4)	66.3 (60.5)	22.1 (22.3)	18.9-26.1 (19.5-26.1)
mEOHP	93.6	<LOD	17.6 (15.9)	100.5 (84.8)	3610.6 (2935.4)	49.4 (42.6)	15.5 (15.7)	13.1-18.5 (13.7-18.5)
mBzP	91.6	<LOD	6.0 (7.0)	38.2 (32.1)	199.4 (132.0)	12.8 (11.2)	6.9 (7.0)	6.1-8.0 (6.2-7.9)
mEHP	72.7	<LOD	7.6 (7.3)	50.1 (47.1)	3401.3 (2765.3)	28.2 (25.3)	7.0 (7.1)	6.0-8.2 (6.1-8.3)
MPB	99.2	<LOD	98.3 (121.9)	3098.4 (3191.5)	67461.3 (46089.3)	1200.7 (1138.8)	102.1 (103.2)	79.8-132.8 (80.5-132.6)
EPB	93.6	<LOD	2.6 (2.9)	120.5 (77.3)	377.5 (146.3)	19.8 (16.0)	3.1 (3.2)	2.5-4.1 (2.5-4.1)
isoPPB	12.0	<LOD	<LOD	0.97 (0.85)	63.9 (51.1)	0.9 (0.9)	NC[b]	NC
nPPB	90.8	<LOD	13.4 (17.5)	685.4 (461.4)	28182.1 (20387.3)	413.4 (365.7)	11.2 (11.3)	8.0-15.4 (8.1-15.4)
isoBPB	26.5	<LOD	<LOD	2.6 (2.3)	59.2 (39.2)	0.6 (0.5)	NC	NC
nBPB	38.6	<LOD	<LOD	28.2 (21.3)	242.3 (148.8)	6.2 (5.1)	NC	NC
BPA	99.6	<LOD	1.2 (1.1)	4.7 (5.6)	144.0 (116.1)	2.6 (2.4)	1.2 (1.2)	1.1-1.4 (1.1-1.4)
239 children, ng of analyte/mL urine (µg of analyte / g creatinine)								
mEP	99.6	<LOD	34.4 (86.6)	230.4 (477.7)	2460.1 (3617.8)	79.2 (182.7)	35.3 (88.5)	30.2-41.0 (76.9-102.0)
miBP	98.7	<LOD	34.4 (101.6)	202.4 (280.6)	886.0 (681.6)	62.4 (123.2)	36.0 (90.3)	31.2-41.4 (81.0-100.8)
mnBP	96.2	<LOD	23.9 (62.3)	162.3 (261.0)	1250.5 (962.0)	47.1 (90.8)	23.3 (58.3)	19.9-27.0 (51.5-65.9)
mEHHP	97.1	<LOD	30.5 (71.0)	158.2 (246.7)	626.3 (1204.3)	51.2 (102.2)	24.9 (62.4)	20.8-29.7 (54.3-71.9)
mEOHP	95.4	<LOD	20.0 (51.0)	116 (171.6)	391.1 (611.1)	35.0 (68.4)	16.9 (42.5)	14.2-20.5 (37.3-48.9)
mBzP	86.2	<LOD	6.5 (17.0)	35.2 (73.5)	241.9 (394.2)	12.9 (27.8)	6.8 (17.0)	5.9-7.8 (15.0-19.3)
mEHP	57.3	<LOD	2.8 (9.1)	23.5 (78.5)	95.01 (203.8)	7.0 (18.2)	3.8 (9.6)	3.4-4.4 (8.5-11.0)
MPB	100.0	<LOD	17.1 (42.6)	942.9 (2710.5)	6805.9 (17014.8)	198.2 (512.2)	25.0 (62.7)	20.0-31.5 (49.5-79.7)
EPB	93.3	<LOD	1.5 (3.7)	96.2 (318.2)	1116.9 (1801.5)	22.0 (66.0)	1.8 (4.5)	1.4-2.4 (3.4-6.0)
isoPPB	2.1	<LOD	<LOD	<LOD	10.8 (13.0)	0.1 (0.4)	NC	NC
nPPB	79.1	<LOD	0.9 (2.3)	111.7 (328.5)	1491.3 (3728.3)	25.7 (73.1)	1.3 (3.2)	0.9-1.7 (2.4-4.4)
isoBPB	6.3	<LOD	<LOD	0.1 (0.4)	1.1 (1.7)	0.1 (0.1)	NC	NC
nBPB	25.9	<LOD	<LOD	1.3 (2.2)	93.2 (665.9)	1.0 (5.9)	NC	NC
BPA	99.6	<LOD	2.1 (5.2)	16.6 (31.0)	68.7 (121.7)	4.5 (9.6)	2.0 (5.0)	1.7-2.4 (4.3-5.8)

[a]<LOD: lower than limit of detection, [b]NC: not calculated

We used unnormalised concentrations because creatinine levels were higher in males (arithetic mean, males: 0.53 g/L, females: 0.45 g/L; independent samples t-test, p-value: 0.043). Independent samples

t-test was utilised to compare creatinine levels since creatinine data were not skewed. Previous studies did not report general differences between sexes (Boas and others 2010; Cutanda and others 2014; Guo and others 2011; Langer and others 2014; Song and others 2013) or have shown slightly higher concentrations in males only for DEHP metabolites (Wittassek and others 2007).

3.1.2 Estimated daily intake

The estimated daily intake levels for all PB and PhE (median values, Table 4) were higher for mothers in pregnancy than for children at about two years of age (Wilcoxon signed ranked test, p-value<0.001), except for DEHP (also higher in mothers but p-value was 0.055), as shown in Figure 1. In contrast, BPA-DI_u was higher in children compared to their mothers (Wilcoxon signed ranked test, p-value 0.013). Although, we must take into account that there is a two and a half years between samplings and except for differentiations in pregnant-child eposure, changes in PhE, PB and BPA market may also affect exposure levels. DEP for mothers and DEHP for children, were the two PhE with the highest median values of DI_u, followed by DiBP, DnBP and BBP in decreasing order. The estimated DI_u of all PhE was higher in mothers compared to children, in contrast to the creatinine-normalised values (Table 5). This is explained by the fact that although creatinine adjustment is a very useful tool to normalise urine density and compare urinary concentration of a homogenous population, it does not take into account that creatinine excretion is dependent on muscle mass and body height (Barr and others 2005b). Furthermore, DEHP DI_u was the highest in children (4.0 µg d^{-1} kg^{-1}, Table 5) and the second highest in mothers (4.4 µg d^{-1} kg^{-1}, Table 4), in contrast to DEHP metabolites levels, which were relatively lower compared to those of other PhE metabolites. This observation is probably due to the extended metabolism of DEHP to many compounds (Koch and others 2005b). The values of Reference Doses (RfD, µg d^{-1} kg^{-1}; DEP:800, DnBP:100, BBP:200, DEHP:20) established by U.S. Environmental Protection Agency (U.S. E.P.A. 2005a; U.S. E.P.A. 2005b; U.S. E.P.A. 2005c) and of Tolerable Daily Intake (TDI, µg d^{-1} kg^{-1}; DEP:500, DnBP:10, BBP:500, DEHP:50) set by the European Food Safety Authority (EFSA 2005), were compared with our results. For DnBP, the 5.9% of mothers revealed higher DnBP-DI_u than DnBP-TDI (0.8% of subjects higher compared to DnBP-RfD). For DEHP, the 6.8% of mother's DI_u exceeded RfD (0.6% exceeded DEHP-TDI). For children, the 1.7% of cases were found exceeding DnBP-TDI. Finally, the 6.3% of children's DEHP-DI_u was higher than the corresponding DEHP-RfD (1.3% of subjects exceeded TDI). For PB daily intake, the same patterns were observed with concentration values (Tables 3 and 4, Figure 1). The higher intake of PB and PhE (except from DEHP) by mothers can be interpreted by the extensive usage of cosmetics (Elder 1984). This was not observed for DEHP, which is widely used as plasticizer (Wormuth

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and others 2006). BPA-Diu was the lowest among the examined endcrine disruptors (median values , Table 4) for both mothers (0.03 µg d^{-1} kg^{-1}) and children (0.05 µg d^{-1} kg^{-1}). Furthemore, none subject of the study exceeded neither current nor recommended BPA-TDI (current: 50 µg d^{-1} kg^{-1} / recommended: 5 µg d^{-1} kg^{-1}) (EFSA 2014).

Table 4. Estimated daily intake of PhE, PB and BPA

|	Minimum	Median	95% percentile	Maximum	Arithmetic mean	Geometric mean	95% CI
239 mothers, (µg d^{-1} kg^{-1})							
DEP	0.2	6.9	74	182.4	17.9	7.1	6.0-8.4
DiBP	NC^c	2.1	11	30.6	3.4	2	1.7-2.3
DnBP	NC	1.9	11.4	4839.8	24	1.7	1.5-2.1
DEHP	NC	4.4	25.6	1015.0	12.2	4.1	3.5-4.8
BBzP	NC	0.3	1.8	9.9	0.6	0.3	0.3-0.4
MPB	NC	500.0	17076.1	388041.8	6388.1	532.7	413.6-688.8
EPB	NC	13.2	599.9	2388.4	109.3	16.4	12.4-21.3
isoPPB	NC	NC	4.0	281.2	4.8	NC	NC
nPPB	NC	73.3	3818.5	162105.5	2203.1	58.2	40.1-82.2
isoBPB	NC	NC	8.1	183.5	2.0	NC	NC
nBPB	NC	NC	82.6	781.4	20.0	NC	NC
BPA	NC	0.03	0.14	4.23	0.08	0.04	0.03-0.04
239 children, (µg d^{-1} kg^{-1})							
DEP	NC	1.4	8.6	91.4	2.9	1.3	1.1-1.5
DiBP	NC	1.4	8.2	36.0	2.5	1.5	1.30-1.7
DnBP	NC	1.0	6.6	50.8	1.9	0.9	0.8-1.1
DEHP	NC	4.0	21.6	69.6	6.8	3.3	2.8-3.9
BBzP	NC	0.2	1.3	9.0	0.5	0.3	0.2-0.3
MPB	NC	66.6	3674.9	26526.7	772.5	97.5	77.5-123.54
EPB	NC	5.8	375.1	4353.3	85.9	7.0	5.4-9.1
isoPPB	NC	NC	NC	42.0	0.5	NC	NC
nPPB	NC	3.4	435.4	5812.6	100.1	5.0	3.7-6.7
isoBPB	NC	NC	0.16	2.6	0.1	NC	NC
nBPB	NC	NC	2.9	217.0	2.3	NC	NC
BPA	NC	0.05	0.37	1.54	0.10	0.04	0.04-0.05

^{c}NC: not calculated
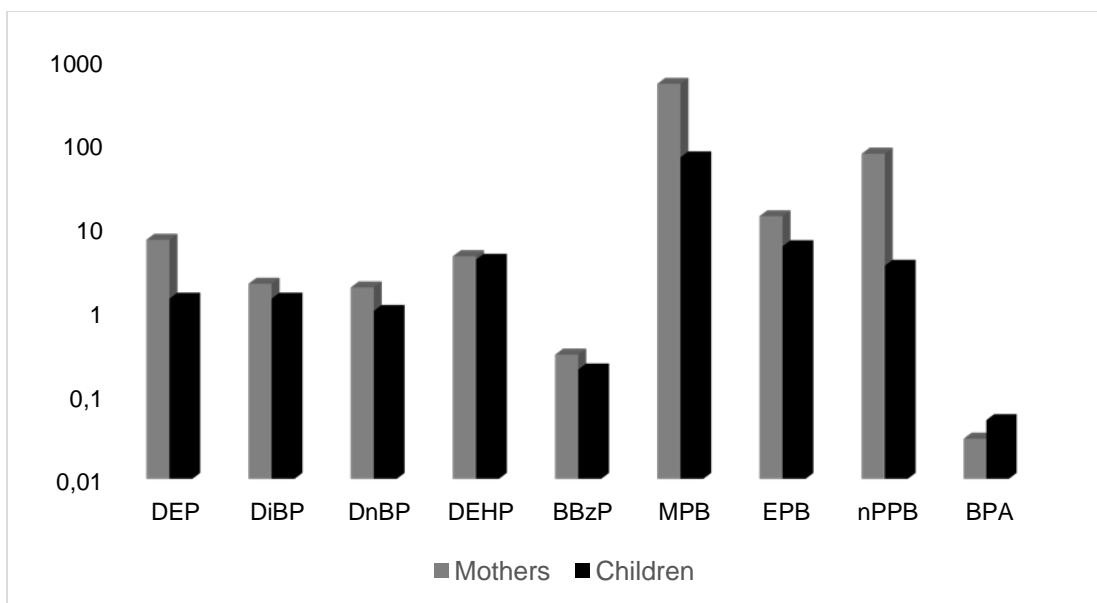
Figure 1. Estimated daily intake mother-child comparison, median values, μg d^{-1} kg^{-1}

3.1.3   Comparison with other studies worldwide

The overall PhE exposure, evaluated in our study, is generally comparable with the literature reports from other countries. Furthermore, the specific PhE exposure pattern is also similar in general. Comparison with other studies (Table 5) demonstrated slightly higher exposure to PhE for mothers in Greece compared to those from Denmark (Tefre de Renzy-Martin and others 2014) and slightly lower compared to those from France (Mortamais and others 2012; Zeman and others 2013). Moreover, for children there are not distinct differences for PhE metabolites levels in general (Table 5). However, a study from Germany (Kasper-Sonnenberg and others 2014) reported the lowest concentrations (except for miBP) levels. More specifically the comparison, presented in Table 5, shows the highest miBP levels in Greece (our study), the highest mnBP levels in Denmark (Boas and others 2010) and the highest mEP levels in USA (Teitelbaum and others 2012).

PB concentration levels for both mothers and children in Greece appear to be clearly higher compared to those reported by a study from Denmark (Frederiksen and others 2013). We have to underline that our data represent only free and glucuronated PB in contrast to the above referred study, which reports total PB concentrations. Finally, children BPA levels in our study were the second higher 5.2 μg/g but the highest levels (Braun and others 2011) were about three times higher compared to ours.

Table 5. Comparison with similar studies, creatinine adjusted median (μg/g) values

Country; number of samples; reference	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	mEHP	MPB	EPB	isoPPB	nPPB	isoBPB	nBPB	BPA
Mothers														
Greece; n=239; this study	132.6	38.7	33.2	24.4	15.9	7.0	7.3	121.9	2.9	<LOD	17.5	<LOD	<LOD	1.1
France; n=287; (Mortamais and others 2012)	106.0	45.7	48.5	-	-	16.0	-	-	-	-	-	-	-	-
Denmark; n=200; (Tefre de Renzy-Martin and others 2014)	18.9	35.3	13.9	5.7	3.72	2.3	1.1	-	-	-	-	-	-	-
France; n=279; (Zeman and others 2013)	34.3	68.7	45.5	44.4	32.9	13.0	17.9	-	-	-	-	-	-	-
Denmark; n=143; (Frederiksen and others 2013)	-	-	-	-	-	-	-	16.0	0.91	<LOD	1.8	<LOD	<LOD	-
Korea; n=757; (Lee and others 2014)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6
USA; n=866; (Quiros-Alcala and others 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1
Spain; n=479 (Casas and others 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2
USA; n=244; (Braun and others 2011)	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2
USA; n=249; (Braun and others 2009)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8
Children														
Greece; n=239; this study	86.6	101.6	62.3	71.0	51.0	17.0	9.1	42.6	3.7	<LOD	2.3	<LOD	<LOD	5.2
Denmark; n=845; (Boas and others 2010)	33.5	-	209.0	52.0	27.0	23.0	6.8	-	-	-	-	-	-	-
USA; n=379; (Teitelbaum and others 2012)	164.9	22.5	68.4	73.9	47.6	41.8	6.4	-	-	-	-	-	-	-
Germany; n=465; (Kasper-Sonnenberg and others 2014)	21.4	41.1	42.3	20.2	13.5	6.0	2.23	-	-	-	-	-	-	1.8
Denmark; n=143; (Frederiksen and others 2013)	-	-	-	-	-	-	-	0.9	0.26	<LOD	<LOD	<LOD	<LOD	-
USA; n=292; (Harley and others 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	3.2
China; n=1089; (Hong and others 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.3
China; n=666; (Wang and others 2014)	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2
USA; n=229; (Braun and others 2011)	-	-	-	-	-	-	-	-	-	-	-	-	-	14.0
USA; n=249; (Braun and others 2009)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.9

It has to be mentioned that bibliographic comparisons suffer from several weaknesses as they don't take into account, market changes of PhE, PB and BPA, corresponding to different time periods of sample collection, different age of children and phase of pregnancy, different methods of analysis and analyte detection limits, lack of statistical tests for inter-study comparisons etc. Especially, for PB levels, more data are needed in order to assess globally their exposure.

3.2 Sources of exposure

3.2.1 Correlation studies

Statistically significant correlations (2-tailed Spearman) were observed between the creatinine adjusted molar concentrations (μmol/g) of the studied compounds both for mothers and children. We only present correlations with p-values lower than 0.01 (Table 6). We thus observed positive correlations between almost all PhE metabolites and BPA for mothers (correlation coefficient, CC: 0.170-0.488). All PB concentration levels were positively correlated (CC: 0.468-0.841) and also correlated with mEP (CC: 0.251-0.335). Likewise, for children almost all studied metabolites correlated positively (CC: 0.167-0.809; Table 6). The observed correlations suggest exposure to mixtures of PB, PhE and BPA. Particularly for mothers, two distinct exposure sources are implied, one containing all study's PhE/BPA and a second with all three studied PB and DEP (whose metabolite is mEP). The DEP/PB relationship suggests cosmetics and personal care products as sources, while PhE/BPA relationships indicate probably plastic and food packaging (Elder 1984; Geens and others 2012; Wormuth and others 2006).

Children age correlated negatively (-0.193 - -0.476) with all studied metabolites concentration levels (Table 6). An interpretation of this observation is that the food exposure ratios (consuming food mass/body mass), the corresponding dermal exposure ratios (skin surface/body mass), and the floor-to-mouth behaviour decrease as children age increases (Casas and others 2011; Wittassek and others 2007). Finally, DEP, DnBP, BBP metabolites and EPB showed weak (0.133-0.225) correlation between mother and children levels, as also observed previously (Cutanda and others 2014). No statistically significant correlations were found for the remaining studied compounds between mothers and children (Table 6). This fact indicates that, although we examined two different individuals within a time interval of ca. three years (sample collection time from women during pregnancy and from their children) implying also changes in PhE-PB-BPA market, some sources of exposure are common for people living together (house, car use etc.). Furthermore, the need for repeated measurements is highlighted since the correlation between mother and children levels is weak.

Table 6. Two-tailed Spearman's correlation coefficients, p-values <0.01

	M-DnBP	M-BBzP	M-DEHP	M-MPB	M-EPB	M-nPPB	M-BPA	C-DEP	C-DiBP	C-DnBP	C-BBzP	C-DEHP	C-MPB	C-EPB	C-nPPB	C-BPA
M[e]-DEP			0.17	0.25	0.34	0.28		0.20								
M-DiBP	0.44	0.35	0.42													
M-DnBP		0.37	0.49							0.23						
M-BBzP			0.44				0.25				0.18					
M-MPB					0.54	0.84										
M-EPB						0.46								0.13		
M-BPA																
C[f]-Age (y)								-0.43	-0.43	-0.48	-0.37	-0.19	-0.38	-0.47	-0.38	-0.21
C-DEP									0.52	0.48	0.38	0.27	0.36	0.40	0.37	0.19
C-DiBP										0.76	0.44	0.42	0.32	0.31	0.34	0.29
C-DnBP											0.58	0.48	0.29	0.34	0.34	0.31
C-BBzP												0.50	0.27	0.31	0.27	0.32
C-DEHP													0.17		0.19	0.30
C-MPB														0.67	0.81	
C-EPB															0.67	0.20

[e]M-: mothers, [f]C-: children

3.2.2   Principal Component Analysis

In order to obtain more information about exposure sources, a PCA was applied to mothers and children metabolite concentration data. For both mothers and children, we perceived two distinct patterns (Table 6), one is mostly due to usage of plastics such as food packaging, toys, car parts, clothing, furniture etc. and the second is usage of personal care-hygiene products and cosmetics (Elder 1984; Geens and others 2012; Wormuth and others 2006). Four factors were retained with Eigen values >1.000 and expressed 66.2% of the variance (Table 7). Mothers were associated with factors 2 and 4 while children were associated with 1 and 3. The factors 1-2 represents combined exposure to PhE/BPA and factors 3-4 exposure to PB /DEP. Also, the study of Spearman correlations conducted to the same results.

Table 7. PCA: rotated component matrix and total variance explained

Component	1	2	3	4
Eigenvalue	4.663	3.274	2.177	1.800
% of Variance	25.9	18.2	12.1	10.0
Cumulative %	25.9	44.1	56.2	66.2
[g]C-DnBP	0.897			
C-DiBP	0.880			
C-DEHP	0.843			
C-BBzP	0.803			
C-DEP	0.662		0.319	
C-MPB			0.899	
C-EPB			0.856	
C-nPPB			0.906	
C-BPA	0.635			
[h]M-DnBP		0.752		
M-DiBP		0.748		
M-DEHP		0.844		
M-BBzP		0.790		
M-DEP		0.448		0.421
M-MPB				0.913
M-EPB				0.740
M-nPPB				0.906
M-BPA		0.509		

Coefficients <0.200 are not presented. Rotation converged in 6 iterations.

[g]C-: children, [h]M-: mothers

3.3 DEHP metabolism

Relative metabolic rates RMR_1 and RMR_2 were calculated for mothers (N=170) and children (total N=136, female and male) for samples in which, all three DEHP metabolites were detected. The RMR_1 arithmetic mean for mothers was 3.33 and for children 8.06. Their difference is statistically significant (independent sample t-test, p-value<0.001). The arithmetic mean RMR_2 for mothers was 0.80 and for children 0.76 respectively, while their difference was not statistically significant (independent sample t-test, p-value 0.156). For mother-child comparisons, independent samples t-test was used since RMR data were not skewed

and we did not considered mothers and children as pairs but as different populations. Male children mean RMR_1 (8.83) was higher from female children (6.67) but without statistically significant difference (independent sample t-test, p-value 0.17). Instead, male children mean RMR_2 (0.72) was significantly lower (p-value 0.03) compared to female children (0.81). Children age with RMR_1 and RMR_2 did not show any statistical significant correlation (two tailed Pearson; p-values: -0.713 for RMR_1 and 0.284 for RMR_2). To sum up: I) the transformation of mEHP to mEHHP is faster in mothers compared to children as was also reported in another study (Song and others 2013). However, other studies (Barr and others 2003; Becker and others 2004; Kasper-Sonnenberg and others 2012; Koch and others 2004b) reported contrasting results; and II) the transformation of mEHHP to mEOHP seems to be faster in male children, indicating that DEHP metabolism is related both with children age/gender and differentiates between mothers and children.

3.4 Conclusions

In our study, the first in Greece and one of the few existing in this scale globally, we observed lower phthalate (except DEHP) and PB daily intake for children than mothers while BPA daily intake was higher in children. Children metabolite levels decreases with age increase. Some sources of exposure seem to be the same in mothers during pregnancy and afterwards. PCA indicated possible sources of PhE, PB and BPA grouped in plastic and personal care-hygiene products for both mothers and children. Male children demonstrated higher concentrations of six PhE metabolites and n-PPB compared to females. DEHP metabolism appears differentiated between mother-child pairs and female-male children. For DiNP exposure, mNP is not a proper biomarker. Our results were comparable with literature reports.

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Appendix 3

Title: Occurrence of bisphenol-A, parabens and phthalate metabolites in urine of preschool-age children in Greece (RHEA cohort) and of phthalates in drinking water and indoor air of the study area

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Abstract

Phthalate esters (PEs), bisphenol-A (BPA) and parabens (PBs), used in numerous consumer products, have endocrine disrupting properties which are linked with health problems. These chemicals, after their insertion into the human body, are metabolised and excreted mainly via urine. The urinary concentration of their metabolites deflects the human exposure to them. We have assessed the exposure to the above chemicals by measuring the urinary concentrations of seven PEs metabolites, six PBs and BPA in five-hundred samples collected from 4-year old children, subjects of the "Rhea" mother-child cohort in Crete, Greece. Di-2-ethylhexyl phthalate (DEHP) metabolism was studied by calculating the Relative Metabolic Rates of its metabolites. The transformation of mono-2-ethyl-5-hexyl phthalate (mEHP) to mono-2-ethyl-5-hydroxy-hexyl phthalate (mEHHP) was negatively correlated with children's age. Exposure to PEs, BPA and PBs was assigned to two main sources: plastic related to PEs and BPA, and personal care-hygiene products to PBs and di-ethyl phthalate. Daily intake calculated for 4-year old children was lower than the corresponding calculated than that calculated in an earlier study of "Rhea" children at 2.5 years. In some cases daily intake levels exceeded the USEPA Tolerable Daily Intake values and the EFSA Reference Doses (e.g. 3.6% of the children exceeded DEHP-Reference Dose). In addition, PEs were determined in drinking water and indoor air of houses and car interiors in the study area. Exposure to PEs, present in indoor air and drinking water, represents a small fraction of the total.

Keywords: exposure, phthalates, parabens, BPA, preschool-age children, Rhea cohort

Highlights

- Urinary levels of seven phthalate metabolites, six parabens and bisphenol-A
- Five-hundred 4-year old children from Rhea Cohort – Greece
- Daily intake is lower compared to 2.5-year old children and during pregnancy
- Air and water phthalate exposure represent a small fraction of the total
- Main sources: Plastic-related and personal hygiene products

Abbreviations: BBP, butyl-benzyl phthalate; BPA, bisphenol-A; CC, correlation coefficient; C_h, average home concentration; C_c, average car concentration; C_u, metabolite concentration, μg/L; C_w, average concentration in water; DEHP, di-2-ethylhexyl phthalate; DEP, di-ethyl phthalate; DiBP, di-iso-butyl phthalate; DI_a, daily intake calculated based on air concentration; DI_u, daily intake based on urinary metabolite levels; levels; DI_w, daily intake based on water concentration levels; DnBP, di-n-butyl phthalate; ED, endocrine disruptors; EHT, elimination half times; EPB, ethyl paraben; HPLC, high performance liquid chromatography; isoBPB, iso-butyl paraben; isoPPB, iso-propyl paraben; F_{ue}, urinary excretion factor; M, average daily water consumption; mBzP, mono-benzyl phthalate; mEHP, mono-2-ethyl-hexyl phthalate; mEHHP, mono-2-ethyl-5-hydroxy-hexyl phthalate; mEOHP, mono-2-ethyl-5-oxo-hexyl phthalate; mEP, mono-ethyl phthalate; mLOD, method limit of

detection; mnBP, mono-n-butyl phthalate; MPB, methyl paraben; MW_1, molecular weight of phthalate diester; MW_2, molecular weight of phthalate metabolite; nBPB, n-butyl-paraben; NC, not calculated; ND, not detected; nPPB, n-propyl paraben; NR, not reported; PBs, parabens; PCA, Principal Component Analysis; PEs, phthalate esters; RfD, reference dose; RMR, relative metabolic rate; RMR_1, mEHHP/mEHP molar concentrations ratio; RMR_2, mEOHP/mEHHP molar concentrations ratio; TDI, tolerable daily intake; W, body weight

Introduction

Endocrine disruptors (EDs) are a group of organic compounds, which cause serious alterations to the normal hormone function in humans and wildlife (World Health Organization, 2012). They interfere with hormone biosynthesis, metabolism or action resulting in a deviation from normal homeostatic control or reproduction in humans (Diamanti-Kandarakis et al., 2009). They disrupt the endocrine system by competing with naturally occurring hormones such as estradiol, or by altering the synthesis and metabolism of these hormones (National Institute of Health, 2010); in addition, there is evidence of reproductive toxicity in laboratory animals and possible health effects in humans (Chapin et al., 2008). Bisphenol-A (BPA), parabens (PBs) and 1,2-diesters of phthalic acids (PEs) are established EDs. Six (6) billion pounds of BPA are produced each year worldwide and over 220,000 pounds of this compound are released yearly into the atmosphere (Burridge, 2003). PEs, with over 18 billion pounds used each year, represent one of the world's high production chemical families (Crinnion, 2010) and PBs, which are used in over 13.200 formulations in nearly all type of cosmetics (Elder, 1984). Human exposure to these chemicals is occurring through the environment, food intake and the use of products containing them, through inhalation, dermal contact and ingestion (ATSDR DEHP, 2001; ATSDR DEP, 1995; ATSDR DnBP, 2001; Meeker, 2010; Soni et al., 2001).

PEs have a variety of common uses. High molecular weight (HMW) PEs are used in plastic as softeners and low molecular weight (LMW) PEs are used in personal care products and pharmaceuticals (Wormuth et al., 2006). Previous animal tests and epidemiological studies have associated exposure to PEs with detrimental effects to reproductive and developmental health, as well as increased risk to cancer (ATSDR DEHP, 2001; ATSDR DEP, 1995; ATSDR DnBP, 2001). PEs normally follow a metabolic pathway in at least two steps, a hydrolysis (phase-I) where the phthalate diester is hydrolysed into the primary metabolite monoester phthalate and is followed (phase-II) by a conjugation in order to form the more hydrophilic glucuronidated metabolite (Calafat et al., 2006).

The 2,2-bis (4-hydroxyphenyl) propane or bisphenol-A (BPA) is used in industry for the production of many pesticides, resins and polycarbonate plastic. BPA can be found in food and beverage processing, and in many products like dental sealants, personal care products, baby bottles, building materials, flame retardant materials and optical lenses, materials for the protection of window glazing, DVDs, and household electronics (Chapin et al., 2008; Geens et al., 2012; Staples et al., 1998). Human exposure to BPA is linked to heart diseases, diabetes, liver abnormalities, reproduction adverse effects and alterations in the thyroid (Rubin, 2011). BPA is excreted mainly via urine in its free form or in its more hydrophilic glucuronide/sulphate conjugate form (Chapin et al., 2008).

PBs is a group of alkyl esters of p-hydroxybenzoic acid. They have low cost of production and demonstrate high chemical stability, inertness, and low acute toxicity (World Health Organization, 2012). These characteristics made them desirable in industry, as antimicrobial preservatives against mould and yeast, in cosmetics, in pharmaceuticals and in food and beverage processing (Elder, 1984). PBs occur also naturally in food, wine, and plants (Soni et al., 2005). *In vitro* studies indicate that PB induce the growth of MCF-7 human breast cancer cells and influence the expression of estrogen dependent genes (Byford et al., 2002). In general, PBs are partially hydrolysed by esterases to p-hydroxy-benzoic acid and produce

glycine/glucuronide/sulphate conjugates, with increased water solubility that are more amenable to urinary excretion than are the free species (Soni et al., 2005; Wang and James, 2006).

In order to assess the exposure of humans, to PEs, PBs and BPA, measurement of the urinary concentration of their metabolites (free species and their conjugates) is essential (Silva et al., 2003; Ye et al., 2006). The elimination half times (EHT) of the above mentioned compounds are low. For example, BPA and DEHP metabolites EHT values are some hours (Koch et al., 2004a; Koch et al., 2005; Volkel et al., 2002). Furthermore, pregnant mothers (their embryos) and children are the most vulnerable populations to endocrine disruptor exposure effects (World Health Organization, 2012). As a consequence, there is need for repeated analyses during pregnancy and early childhood in order to assess exposure levels and possible health outcomes.

In a previous study (Myridakis, 2015a) we measured concentration levels of seven PEs metabolites, six PBs and BPA in urine samples of two hundred and thirty-nine (239) mother-child pairs (6th month of pregnancy / 2.5 years) of the Rhea cohort (Chatzi et al., 2009). In this study we assessed the urine levels of the above-mentioned metabolites of five hundred (500) 4-year old children, also subjects of the Rhea cohort. Furthermore, we assessed PEs levels in indoor air and drinking water in area of Heraklion. In the present study we: I) evaluated the exposure to the study chemicals in a larger population of preschool-age children, II) estimated the daily intake (DI) of PEs, PBs and BPA, III) assessed the patterns of the exposure IV) obtained an overview of childhood exposure in Greece, V) investigated the contribution of PEs exposure through water - air inhalation and VI) compared our data with other relevant studies worldwide.

Materials and methods

Study population

The present study is part of the "Rhea" project, a pregnancy cohort which examines prospectively a population-based cohort of pregnant women and their children at the prefecture of Heraklion, Crete, Greece (Chatzi et al., 2009; Patelarou et al., 2011). Briefly, women who became pregnant during February 2007-February 2008 participated in the study. Women, residents of the study area, >16 years of age, completed face-to-face interviews and provided blood and urine samples, visiting a participating hospital or private clinic during the 10th–13th week of gestation. The next contacts with the mothers were at 24 weeks of gestation, at birth, at 8-10 weeks after delivery and for child's follow-up at 9th, 18th months, and at 4 years of age. Of 1363 singleton live births in the Rhea study, 879 children participated at the 4 years follow up, during which urine samples were obtained from 800 children. Of them, a random subset of 500 children (221 females-279 males, 4.24 ± 0.24 years old; mean age ± standard deviation) was included in the present analysis. The study was approved by the Ethical Committee of the University Hospital of Heraklion (Crete, Greece) and all participants provided written informed consent.

Urine samples were collected in urine boxes and stored at 4°C until procession. Within 4 hours, samples were aliquoted in 4mL cryovials and stored at -80°C. Urine boxes and cryovials were made of polypropylene and checked for possible contaminations. Creatinine levels were 0.70±0.36 g/L (arithmetic

mean ± standard deviation). Exclusion criteria were set out of 0.1-3.0 range. We did not apply the commonly used exclusion criteria (0.3-3.0 g/L), because creatinine values below 0.3 mg/L in children do not necessarily indicate excessive dilution but are indicative of lower muscle mass compared to adults (Koch et al., 2011).

Environmental samples collection

Ten houses were selected in Heraklion area (study area of the Rhea cohort) for monitoring phthalate esters indoor air levels. Moreover the air concentrations of PEs were determined in the interior of ten used private cars of Heraklion citizens. Indoor air sampling was conducted with a Buck sampling pump (flow rate: 5L/min; duration: 9-18h; Sigma Aldrich, USA) equipped with polyurethane foam filters (diameter: 5.5 cm; height: 8 cm) during April-July 2011. Moreover forty-nine tap water samples were collected from November 2013 up to September 2014 also in the study area of the Rhea cohort (Goslan et al., 2014). Water samples were collected in 250-ml glass bottles with glass caps, during the morning in order to monitor PEs levels in water, which had remained several hours inside the in-home water network. Sampling was performed from November 2013 up to September 2014,

Instrumental analysis

An aliquot of each urine sample (1 mL) was analysed for seven PEs metabolites, six PBs and BPA (Table 1) using an analytical protocol previously described (Myridakis et al., 2015b). Method limits of detection of studied urinary metabolites are depicted in Table 1. Samples exceeding the upper limit of linearity (512 ng/mL) were reanalysed, diluted with nanopure water. Two quality controls samples (spiked pooled urine) and two blank samples (synthetic urine) were analysed with every forty six (46) urine samples. A second aliquot of 0.5 mL urine was analysed for creatinine concentration using the OLYMPUS 2700 immunoassay system (Beckman Coulter, USA). All samples were measured in duplicates. The amount of each sample was quantified by the standard curve performed in each assay.

Five phthalate diesters (parent compounds of the urinary phthalate metabolites) were determined in tap water and indoor air. Their mLODs are reported in Table 1. The procedure for the water analysis was based on a previously described protocol (Environmental Protection Agency, 1995) slightly modified as follows: 100 mL of water were spiked with surrogate standard (deuterated-DEHP) and liquid-liquid extracted three times with 5 mL dichloromethane (15 mL total). Extracts were then loaded onto a Pasteur pipette filled with Na_2SO_4 (5g, anhydrous) in order to remove water residues. At a next step dried extracts were concentrated to 100 μl with a vacuum rotational evaporator, followed by a gentle N_2 stream. The samples were spiked with internal standard (benzyl benzoate) and finally were analysed with GC-MS.

Linearity was excellent (R^2>0.99, 0.6-50 μg/mL), recoveries (n=5) ranged from 63 to 110%; repeatability tests at two different levels (800 and 10000 ng/L) showed standard deviation <3.44%. Average blank contamination was: DEP 1.6 ng; DiBP 3.9 ng; DnBP 2.1 ng; DEHP 22.9 ng; BBP <LOD, (standard deviation < 11.5%). In every four water samples, a blank sample was also analysed. For the analysis of indoor

air samples, polyurethane foams were spiked with surrogate standard (deuterated-DEHP) and processed using accelerated solvent extraction (oven temperature 90°C; pressure: 1500psi; heating time: 5min; static time: 5min; number of cycles: 1; flush volume: 60%; purge time: 1min). The extracts were condensed to 500 µL with a vacuum rotational evaporator. Then with a gentle N_2 stream were condensed to 100 µL, were spiked with internal standard (benzyl benzoate) and finally were analysed with GC-MS. Linearity was excellent (R^2>0.99, 0.6-50 µg/mL) and recoveries (n=5) ranged from 73.3% to 90.3% (standard deviation < 12.3%). Blank contamination was: DEP 9.6 ng; DiBP 33.8 ng; DnBP 22.2 ng; DEHP 82.4 ng; BBP 14.0, <82.4 ng (standard deviation <33% except BBP with 107%). In every five air samples, a blank sample was also analysed. The GC-MS system was consisted of an Agilent GC 6890N/MSD 5973 equipped with an Autosampler HP 7683. The GC-MS parameters were as follows: 1 µL on-column injection; capillary column DB-5MS (30 m, 0.25 mm i.d., 0.25 µm film thickness); carrier gas helium; constant velocity 33 cm/s; transfer line 290°C; temperature program: initial temperature 60° C, 20° C/min to 180° C, 10° C/min to 290 ° C, held for 10 min, total duration 27 min; electron impact at 70 eV; selected ion monitoring mode.

Statistical analysis

Statistical analysis was performed with the software SPSS 22.0 (IBM Corporation, U.S.A.). Measurements below mLOD (not detected) were substituted by the mLOD divided by the square root of 2 (two) (Hornung and Reed, 1990), as the most widely used way to handle non-detects urinary metabolites studies (Ferguson et al., 2014; Song et al., 2013). Arithmetic mean, minimum, median, 95th percentile, maximum, geometric mean and 95% confidence interval of geometric mean (95 % CI) values were calculated for both unadjusted/creatinine-adjusted urinary concentrations and estimated daily intake data.

The daily intake of the studied EDs was estimated by adapting a commonly used toxicokinetic model to our data (Equation 1) (Beko et al., 2013; Dirtu et al., 2013; Ma et al., 2013), where: DI_u (Daily Intake calculated using urinary metabolites, µg×d^{-1}×kg^{-1} of body weight), C_u (metabolite concentration, µg/L), F_{ue} (urinary excretion factor, molar ratio of parent compound in taken to metabolite excreted), MW_1 (molecular weight of PE, g/mol), MW_2 (molecular weight of PE metabolite, g/mol) and W (body weight, kg). Especially for PBs and BPA ratio (MW_1/MW_2) was set equal to 1.

F_{ue} values for mEHHP and mEOHP were taken from (Koch et al., 2004a) and (Koch et al., 2005), for mBzP and mnBP from (Anderson et al., 2001), for PBs from (Ma et al., 2013) and since for miBP and mEP, F_{ue} values were not available, we used the same with mnBP. V_u considered 0.0224 L/kg body weight for children (Miller and Stapleton, 1989; Szabo and Fegyverneki, 1995). We chose to use a value for children volume urine, which doesn't take into account body weight when it is applied to our toxicokinetic model, because children grow rapidly and a stable volume of urine as in used mothers could introduce uncertainty in daily intake estimation.

Equation 1: $DI_u = \frac{C_u \times V_u \times MW_1}{W \times F_{ue} \times MW_2}$ (µg of ED $\times$ d^{-1} $\times$ kg^{-1} of body weight)

Equation 2: $DI_a = \frac{[(C_h \times 0.945) + (C_c \times 0.055)] \times 8.3}{W}$ (µg of ED $\times$ d^{-1} $\times$ kg^{-1} of body weight)

Equation 3: $DI_w = \frac{C_w \times M}{W}$ (µg of ED × d^{-1} × kg^{-1} of body weight)

Furthermore, the daily intakes from air (DI$_a$) and water (DI$_w$) were estimated using Equations 2 and 3 respectively. Since air and water concentration data were not available for each cohort subject, we used the average air and water levels in Heraklion area for our calculations. Especially, for DI$_a$ (Equation 2), we supposed that a children (average body weight of children participating in this study, W:18.4 kg) spends 22.5 h (95.5%) daily indoor and ≈1.5 h (5.5%) in car (C$_h$: average home concentration; C$_c$: average car concentration) and inhales 8.3 m^3/d (Wilson et al., 2001); for DI$_w$, the average concentration in water (C$_w$), the average body weight as in Equation 2 and an average daily water consumption (M: 1L/d) (World Health Organisation, 2008).

In order to investigate possible differentiations in DEHP metabolism among population groups, relative metabolic rate (RMR) of DEHP was calculated as described in literature (Boas et al., 2010; Song et al., 2013). Briefly, RMR$_1$ (1st step of metabolism) considered as the molar concentration ratio of mEHP/mEHHP and RMR$_2$ (2nd step) the ratio of mEHHP/mEOHP. Since all three metabolites of DEHP detected in 100% of the samples we didn't exclude any sample from the RMR calculation. For two-tailed Spearman correlations, urinary molar (nmol/mL) concentrations were used. For two-tailed Pearson correlations, unnormalised RMR values were used since RMR data were not skewed.

Principal component analysis (PCA) with Kaiser normalisation, Mann-Whitney U (concentration levels gender-based comparison) and Wilcoxon signed ranked (comparison of the same children at 2.5 and 4 years) tests were applied to unadjusted for creatinine, log10 transformed concentrations. Independent samples t-test was applied to unnormalised RMR data (mothers-children as independent populations and male-female children comparisons) and to gender-based creatinine levels comparison. For PCA and correlation studies, molar concentration levels were used and mEHHP-mEOHP concentrations summed as DEHP metabolites.

Analytes with detectability lower than 50.0% (isoPPB, isoBPB and nBPB) were excluded from geometric mean calculation and correlation studies. For PCA analysis, isoPPB and isoBPB were excluded (detectability: 3.8% and 10.0% respectively) while nBPB was included (detectability: 37.6%). DEHP-DI$_u$ considered as the arithmetic mean of DI$_u$ for mEHHP and mEOHP. mEHP was excluded from the above analyses (PCA, correlation studies, DI$_u$) due to its relatively lower levels in urine and shorter half-life compared to the other two measured DEHP metabolites, mEHHP and mEOHP (Frederiksen et al., 2007; Koch et al., 2005; Silva et al., 2006a; Silva et al., 2006b; Wittassek and Angerer, 2008).

Comparison with other studies

We performed literature search for similar studies via EndNote X7 (Thompson Reuters) in PubMed database on January 13, 2015. The search criteria were the following: for PE metabolites/BPA, titles containing (*phthalate or bisphenol-a or bpa) and (child*) and for PBs titles containing *paraben*. The selection

criteria were: for studies measuring total metabolites (free, glucuronated and sulphated) from children for over 200 urine samples for PE metabolites/BPA and 100 for PBs, with creatinine normalised median concentrations available for BPA or at least for 4 common PEs metabolites or 4 common PBs with our study.

Initially, 585 articles were identified: 96 hits for (*phthalate* and child*), 55 for (bisphenol-a and child*), 4 for (bpa and child*) and 430 for *paraben*. The same with our previous search, fifteen (14) of them fulfilled the search criteria: (Boas et al., 2010; Braun et al., 2011; Braun et al., 2009; Casas et al., 2013; Frederiksen et al., 2013; Harley et al., 2013; Hong et al., 2013; Kasper-Sonnenberg et al., 2014; Lee et al., 2014; Mortamais et al., 2012; Quiros-Alcala et al., 2013; Tefre de Renzy-Martin et al., 2014; Teitelbaum et al., 2012; Wang et al., 2014; Zeman et al., 2013). References of the selected papers were also checked but no additional articles identified. In case of concentrations given separately for male/female children, the arithmetic mean of the given median values was used.

4. Results and discussion

Concentration levels in urine

PEs metabolites levels for both unadjusted and creatinine adjusted concentrations are presented in Table 2 while PBs and BPA levels are depicted in Table 3. Detectability of Pes metabolites were >93.4%. DEHP metabolites and mEP were detected in all samples. MPB, EPB and nPPB were below mLOD at >92.6%. nBPB was detected in 37.6% of the samples while isoPPB and isoBPB were detected with lower rate (3.8% and 10% respectively). Finally, BPA detected in almost all samples (98.8%). Based on creatinine adjusted median concentration levels, mEP was the most abundant PEs metabolite followed in decreasing order by miBP, mEHHP, mEOHP, mnBP, mEHP and mBzP (Table 2). Furthermore, concerning about PBs, creatinine normalised median levels of MPB were the highest (17.6 µg/g) among the examined PBs, EPB and nPPB were in the same levels (1.5 µg/g). The other three PBs, when we examined their creatinine adjusted arithmetic mean (their detectability were <50% therefore median could not be calculated) levels showed that the most abundant was nBPB (1.0 µg/g) followed by isoBPB and isoPPB (0.2 and 0.1 µg/g respectively). BPA creatinine adjusted median levels were at 1.9 µg/g (Table 3). No statistically significant (p-value < 0.05) difference observed in any metabolite levels (unnormalised) between male and female children in contrast with our previous study (Myridakis, 2015a) for 2.5 years of age where males had exhibited higher concentrations for nPPB and all examined PEs metabolites except from mEHP. We chose to use unnormalised concentrations because creatinine levels were higher in males (arithetic mean, males: 0.74 g/L, females: 0.65 g/L; independent samples t-test, p-value: 0.009). An analogous difference had observed in 2.5 year children creatinine levels (Myridakis, 2015a). Analogous studies show controversial reuslts about relation of gender with children with metabolite levels, a fact which denotes influence of more parameters, for e.g. age (Boas et al., 2010; Cutanda et al., 2014; Guo et al., 2011; Langer et al., 2014; Song et al., 2013; Wittassek et al., 2007; Zhang et al., 2014).

The overall levels of all studies metabolites are relatively lower compared to our previous study (Myridakis, 2015a) at 2.5-year old children (Table 4). Furthermore both of our studies are generally comparable with the literature reports from other countries. The specific PEs exposure pattern is also similar in general. There are not distinct differences for PEs metabolites levels in general (Table 4). However, our study exhibited the highest miBP and the lowest mnBP levels. This fact may be explained by the gradual replacement of DnBP (parent compound of mnBP) with DiBP (parent compound of miBP) (Table 1) and the point that our study is the most recent (Wittasek et al 2007). Concerning about PBs, although concentration levels were lower at 4-year children compared to 2.5-year, in both cases they appeared to be clearly higher compared to a study from Denmark (Frederiksen et al., 2013). Finally, BPA levels were average in relation to the other available reports (Table 4).

DEHP metabolism

We calculated the relative metabolic rates RMR_1 and RMR_2 for all samples (N=500) since DEHP metabolites (mEHP, mEHHP, mEOHP) were in detected in all samples. The arithmetic mean of RMR_1 was 5.11 and RMR_2 0.85. No gender-based differences were observed when we applied independent samples t-test neither for RMR_1 nor for RMR_2. We compared RMRs in 4-year children with 2.5-year. We didn't not compared with pregnant women due to heterogeneity among population groups. The results are depicted in Figure S1 of Supplementary data. The RMR_1 in 4-year children is statistically significantly lower (independent samples t-test, p-value<0.001) compared to 2.5-year children (RMR_1: 8.06). RMR_2 was in comparable levels for 4-year (0.85) and 2.5-year (0.76) children. To conclude, the transformation of mEHP to mEHHP (as expressed by RMR_1) seems to be negatively related with age, an observation also reported by Song et al. (Song et al., 2013). However, other studies (Barr et al., 2003; Becker et al., 2004; Kasper-Sonnenberg et al., 2012; Koch et al., 2004b) reported contrasting results.

Patterns of exposure

Spearman correlations (2-tailed) were applied to concentration data of the studied compounds (Table S1; Supplementary data). Negative correlation with age was observed only for BPA (-0.176, p-value<0.01) in contrast with our previous study where all studied compounds were correlated. This observation can be interpreted by the fact that the factors, which affect exposure in relation with age (consuming food mass/body mass; skin surface/body mass; floor-to-mouth behaviour decrease as children age increases (Casas et al., 2011; Wittassek et al., 2007)) change less in higher children ages. All examined compounds correlated positively (0.247 - 0.699, p-value<0.01), a point that indicates combined exposure, although further analysis is required to trace more specific mixtures. For this reason, we applied a PCA and the results are depicted in Figure 1 and in Table S2 of the Supplementary data. Two factors were retained with Eigen values over 1.000 and expressed 54.96% of the variance. The first factor indicates that PBs exposure is combined furthermore DEP is present in these mixtures since all PBs and DEP are correlated positively. The second factor denotes that BPA and PEs are correlated and the exposure to them is also combined. The PBs-DEP mixtures are possibly from usage of personal care-hygiene products and the PEs-BPA from plastic usage (food packaging,

toys, car parts, clothing, furniture etc.) (Elder, 1984; Geens et al., 2012; Wormuth et al., 2006). The same distinct patterns were also perceived in 2.5-year children and during pregnancy (Myridakis, 2015a).

Estimated daily intake based on urinary metabolites, indoor air and drinking water concentration levels

Based on median DI levels, the highest PEs-DI was exhibited by DEHP (4.02 µg d^{-1} kg^{-1}) followed by DnBP, DiBP and DEP (0.70 – 1.30 µg d^{-1} kg^{-1}). The DI of BBP was in relatively lower levels (0.17 µg d^{-1} kg^{-1}) (Table 5). Regarding about PBs, MPB had the highest DI (25.75 µg d^{-1} kg^{-1}) with EPB and nPPB at comparable levels (1.93 - 2.01 µg d^{-1} kg^{-1}). BPA showed the lowest median DI (0.026 µg d^{-1} kg^{-1}) (Table 5). The values of Reference Doses (RfD, µg d^{-1} kg^{-1}; DEP: 800, DnBP: 100, BBP: 200, DEHP: 20, BPA: 50) established by U.S. Environmental Protection Agency (U.S. Environmental Protection Agency, 2005a; U.S. Environmental Protection Agency, 2005b; U.S. Environmental Protection Agency, 2005c) and of Tolerable Daily Intake (TDI, µg d^{-1} kg^{-1}; DEP:500, DnBP:10, BBP:500, DEHP:50, BPA: 50 (established) / 5 (temporary)) set by the European Food Safety Authority (European Food Safety Administration, 2005), were compared with our results. For RfD, 3.6% of the children exceeded DEHP-RfD (20 µg d^{-1} kg^{-1}). For TDI, 0.2% exceeded DEP-TDI, 0.4% DiBP-TDI, 1% exceeded DnBP-TDI and 1% DEHP-TDI. BPA-DI didn't exceed even the strictest proposed-TDI (4 µg d^{-1} kg^{-1}) in any case.

DI levels for 4-years old children were compared with those for 2.5-years old children (Myridakis, 2015a) as individual populations, using Mann-Whitney U test. As can be seen in Figure S2 of Supplementary data, 4-year old children displayed the lowest DI for all examined chemicals. This difference is lower vs 2.5-year old children for all studied compounds (p-value < 0.003) except DEP and DEHP. We tested also the differences between only the paired samples at 2.5-year and 4-year (Wilcoxon signed ranked test) and the results were similar. To conclude, there is general trend in most cases which shows that DI is higher in 2.5-years old children compared to 4-years old. This trend aids the fact that early childhood is the most vulnerable life period to endocrine disruptor exposure (World Health Organization, 2012) due to higher exposure except from increased human organism susceptibility. Although, it must be taken into account the changes/restrictions in PEs, BPA (European Food Safety Administration, 2015) and PBs market probably have lowered/altered the exposure through last years and therefore, between sampling periods.

PEs concentration levels in water, indoor air (homes and cars) and the estimated corresponding daily intakes (from water, air and from all sources calculated through urine concentrations) are presented in Table 6. Home and car air showed comparable concentration patterns, with DEP being the most abundant PE, followed by DiBP, DnBP/DEHP and BBP. For drinking water, DiBP showed the highest levels followed by DnBP, DEP and DEHP. BBP was not detected in water samples. The estimated DI_w and DI_a indicated that intake though air inhalation is generally higher compared to water ingestion. However, both of these routes of exposure represent a small fraction of the total PEs exposure, expressed by DI_u. Although the different periods of sampling for air, water and urine may limit the accuracy of our estimations, we consider that the differences in DI levels are very important to contradict our observations.

Conclusions

In this study, we assessed the exposure levels of PEs, PBs and BPA in five hundred (500) 4-year old children (subjects of Rhea cohort). No gender differentiation was observed concerning metabolite levels. DI at 4-year old children was lower than the corresponding of 2.5-year old children. Comparison with other studies worldwide did not reveal strong differentiations in concentration levels for PEs metabolites and BPA. For PBs there is need for more studies to proceed to solid conclusions. PCA grouped the exposure to two distinct sources: plastic for PEs-BPA and personal care-hygiene products for PBs-DEP; the same pattern was observed in 2.5-year children and during pregnancy. The rate of mEHP transformation to mEHHP (a phase of DEHP metabolism) seems to be age-related. The determination of PEs in drinking water and indoor air, in order to evaluate their corresponding exposure, revealed their low input to the total exposure, evaluated through the metabolites urine concentrations.

Acknowledgements

Tables

Table 1. Studied endocrine disruptors and their method limits of detection (mLOD)

Parent compounds in air and water	Metabolites in urine	Method limit of detection (mLOD)		
		ng/mL urine	ng/m³ air	pg/mL water
di-ethyl phthalate (DEP)	mono-ethyl phthalate (mEP)	0.40	0.02	6.7
di-n-butyl phthalate (DiBP)	mono-n-butyl phthalate (mnBP)	0.25	0.02	9.1
di-iso-butyl phthalate (DnBP)	mono-iso-butyl phthalate (miBP)	0.41	0.02	5.6
di-2-ethylhexyl phthalate (DEHP)	mono-2-ethylhexyl phthalate (mEHP)	0.84	0.05	53.7
	mono-2-ethyl-5-hydroxy-hexyl phthalate (mEHHP)	0.01		
	mono-2-ethyl-5-oxo-hexyl phthalate (mEOHP)	0.18		
butyl-benzyl phthalate (BBP)	mono-benzyl phthalate (mBzP)	0.02	0.13	4.1
methyl paraben (MPB)		0.06	-	-
ethyl paraben (EPB)		0.06	-	-
iso-propyl paraben (isoPPB)		0.13	-	-
n-propyl paraben (nPPB)		0.09	-	-
iso-butyl paraben (isoBPB)		0.04	-	-
n-butyl paraben (nBPB)		0.04	-	-
Bisphenol-A (BPA)		0.01	-	-

Table 2. Descriptive statistics of PE metabolite levels, ng/mL urine (μg/g creatinine)

PEs metabolite	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	mEHP
Arithmetic mean	131.6	43.3	32.7	44.3	36.4	10.1	10.6
	(159.5)	(62.5)	(45.3)	(67.4)	(54.5)	(14.3)	(17.2)
Geometric Mean	38.0	24.8	13.1	26.3	21.0	4.4	6.7
	(62.7)	(40.9)	(21.6)	(43.3)	(34.6)	(7.3)	(11.1)
Geometric Mean 95% CI	34.1-42.3	22.2-27.8	11.3-15.2	24.0-28.7	19.1-23.0	4.0-5.0	6.2-7.3
	(57.1-68.9)	(36.9-45.4)	(18.9-24.7)	(40.3-46.6)	(32.0-37.3)	(6.6-8.1)	(10.3-11.9)
Minimum	1.0	<mLOD	<mLOD	0.2	0.4	<mLOD	1.1
	(0.9)			(0.2)	(0.4)		(1.7)
Median	34.9	29.5	17.2	27.4	22.6	4.5	6.2
	(53.5)	(48.8)	(27.9)	(40.7)	(35.4)	(7.0)	(10.5)
95th percentile	293.1	130.5	104.4	124.5	103.7	35.3	34.4
	(416.0)	(158.2)	(128.8)	(161.7)	(124.5)	(50.6)	(46.5)
Maximum	19549.1	586.3	564.4	1504.9	1107.3	426.5	141.1
	(17611.8)	(671.4)	(695.0)	(2246.1)	(1652.7)	(313.6)	(300.7)
Detected (%>mLOD)	100.0	96.8	93.4	100.0	100.0	99.0	100.0

Table 3. Descriptive statistics of PBs and BPA levels, ng/mL urine (µg/g creatinine)

Phenol	MPB	EPB	iso PPB	nPPB	isoBPB	nBPB	BPA
Arithmetic mean	79.6 (147.2)	7.7 (12.0)	0.1 (0.1)	10.0 (17.2)	0.2 (0.2)	0.6 (1.0)	2.0 (3.2)
Geometric Mean	15.1 (24.9)	1.1 (1.9)	NC	1.1 (1.9)	NC	NC	1.1 (1.7)
Geometric Mean 95% CI	13.3-17.2 (22.0-28.3)	1.0-1.3 (1.6-2.1)	NC	1.0-1.3 (1.6-2.2)	NC	NC	1.0-1.2 (1.6-1.9)
Minimum	0.7 (0.8)	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD	<mLOD
Median	11.5 (17.6)	0.9 (1.5)	<mLOD	0.9 (1.5)	<mLOD	<mLOD	1.2 (1.9)
95th percentile	263.3 (424.7)	25.5 (43.5)	<mLOD	34.3 (49.3)	0.1 (0.2)	0.7 (1.4)	6.4 (10.0)
Maximum	3846.7 (11039.7)	315.2 (573.1)	15.0 (14.5)	483.2 (1098.1)	50.4 (45.4)	97.1 (158.9)	59.2 (67.3)
Detected (%>mLOD)	100.0	96.8	3.8	92.6	10.0	37.6	98.8

NC: Not Calculated

Table 4. Comparison with similar studies, creatinine adjusted median (μg/g) values

Country; number of samples; age (y); reference	mEP	miBP	mnBP	mEHHP	mEOHP	mBzP	mEHP	MPB	EPB	isoPPB	nPPB	isoBPB	nBPB	BPA
Present study; Greece; n=500	53.5	48.8	27.9	40.7	35.4	7.0	10.6	17.6	1.5	<mLOD	1.5	<mLOD	<mLOD	1.9
Previous Rhea study, 2.5-year children; Greece; n=239;	86.6	101.6	62.3	71.0	51.0	17.0	9.1	42.6	3.7	<LOD	2.3	<LOD	<LOD	5.2
Denmark; n=845; 4-9 y; (Boas et al., 2010)	33.5	-	209.0	52.0	27.0	23.0	6.8	-	-	-	-	-	-	-
USA; n=379; 7.3 y; (Teitelbaum et al., 2012)	164.9	22.5	68.4	73.9	47.6	41.8	6.4	-	-	-	-	-	-	-
Germany; n=465; 8-10 y; (Kasper-Sonnenberg et al., 2014)	21.4	41.1	42.3	20.2	13.5	6.0	2.23	-	-	-	-	-	-	1.8
Denmark; n=143; 6-11 y; (Frederiksen et al., 2013)	-	-	-	-	-	-	-	0.9	0.26	<LOD	<LOD	<LOD	<LOD	-
USA; n=292; 5 y; (Harley et al., 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	3.2
China; n=1089; 9 y; (Hong et al., 2013)	-	-	-	-	-	-	-	-	-	-	-	-	-	1.3
China; n=666; 9-12 y; (Wang et al., 2014)	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2
USA; n=229; 1-3 y; (Braun et al., 2011)	-	-	-	-	-	-	-	-	-	-	-	-	-	14.0

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Table 5. Estimated daily intake of PEs, PBs and BPA (µg d^{-1} kg^{-1})

	Arithmetic mean	Geometric mean	Geometric mean 95% CI	Minimum	Median	95% percentile	Maximum
DEP	4.89	1.41	1.27-1.57	0.04	1.30	10.89	726.23
DiBP	1.76	1.01	0.90-1.13	<mLOD	1.20	5.31	23.84
DnBP	1.33	0.53	0.46-0.62	<mLOD	0.70	4.25	22.94
BBP	0.38	0.17	0.15-0.19	<mLOD	0.17	1.32	15.95
DEHP	6.48	3.83	3.50-4.18	0.06	4.02	17.30	206.92
MPB	178.34	33.88	29.78-38.54	1.52	25.75	589.88	8616.56
EPB	17.15	2.52	2.18-2.91	<mLOD	2.01	57.23	706.04
isoPPB	0.32	NC	NC	<mLOD	<mLOD	<mLOD	33.50
nPPB	22.41	2.55	2.16-3.00	<mLOD	1.93	76.74	1082.29
isoBPB	0.54	NC	NC	<mLOD	<mLOD	0.31	112.85
nBPB	1.43	NC	NC	<mLOD	<mLOD	1.48	217.56
BPA	0.045	0.024	0.021	<mLOD	0.026	0.143	1.327

NC: not calculated

Table 6. Concentration levels of PEs in indoor air and tap water and corresponding daily intake compared with urinary daily intake, average values.

PEs	Home air concentration (ng/m^3)	Car interior air concentration (ng/m^3)	Tap water concentration pg/mL	Air daily intake (DI$_a$) (µg d^{-1} kg^{-1})	Water daily intake (DI$_w$) (µg d^{-1} kg^{-1})	Total daily intake 4-year old children (DI$_u$) (µg d^{-1} kg^{-1})
DEP	1705.1	2459.6	574.6	0.733	0.031	4.888
DiBP	944.6	759.5	2058.6	0.405	0.112	1.762
DnBP	467.6	235.8	1165.4	0.200	0.063	1.329
BBP	5.8	7.3	<LOD	0.002	NC	0.379
DEHP	178.2	350.4	415.9	0.077	0.023	6.475

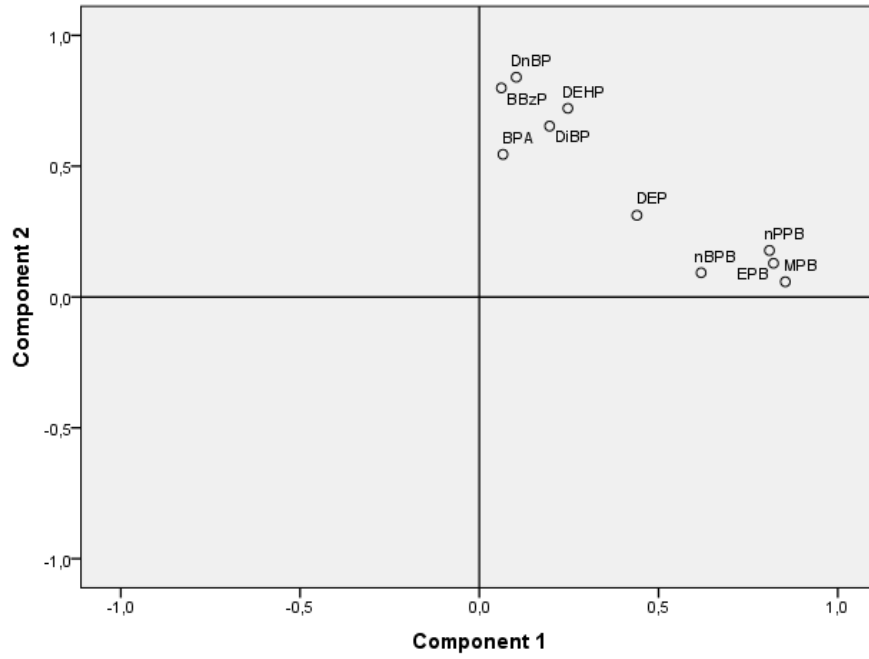NC: not calculated

Figures

Figure 1. Principal components plot

Supplementary data

Table S1. Two-tailed Spearman's correlation coefficients, p-values <0.01

	DiBP	DnBP	BBP	DEHP	MPB	EPB	nPPB	BPA
Age (y)								-0.176
DEP	0.463	0.333	0.332	0.494	0.453	0.367	0.409	0.274
DiBP		0.685	0.634	0.614	0.371	0.307	0.387	0.447
DnBP			0.699	0.629	0.310	0.268	0.334	0.508
BBP				0.637	0.292	0.247	0.349	0.395
DEHP					0.395	0.343	0.411	0.454
MPB						0.620	0.675	0.273
EPB							0.569	0.301
nPPB								0.293

Table S2. PCA: rotated component matrix and total variance explained

Component	1	2
Eigenvalue	3.72	1.78
% of Variance	37.20	17.76
Cumulative %	54.96	37.20
DnBP	0.10	0.84
BBP		0.80
DEHP	0.25	0.72
DiBP	0.20	0.65
BPA		0.55
DEP	0.44	0.31
MPB	0.85	
EPB	0.82	0.13
nPPB	0.81	0.18
nBPB	0.62	

Coefficients <0.1 are not presented. Rotation converged in 3 iterations
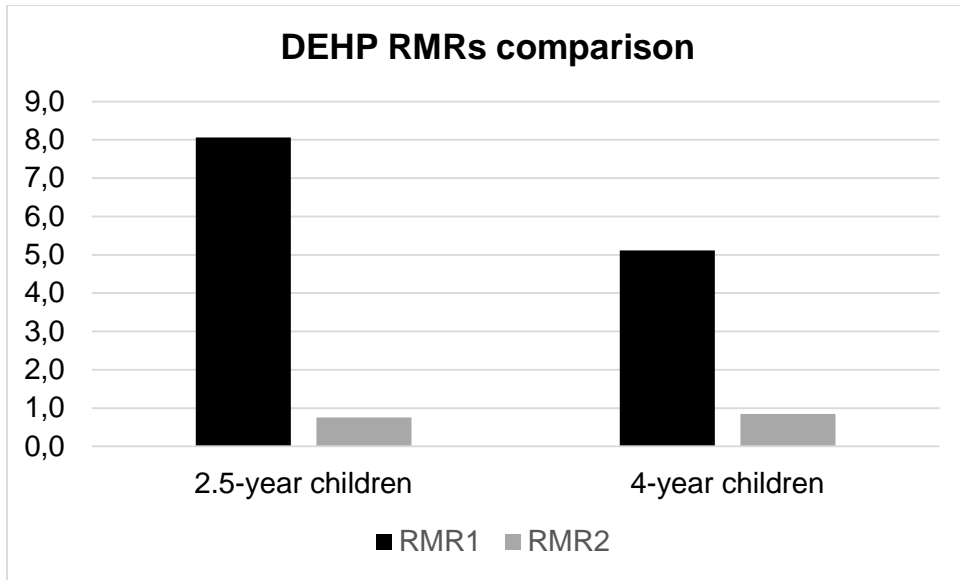
Figure S1. DEHP relative metabolic rates comparison, arithmetic mean values

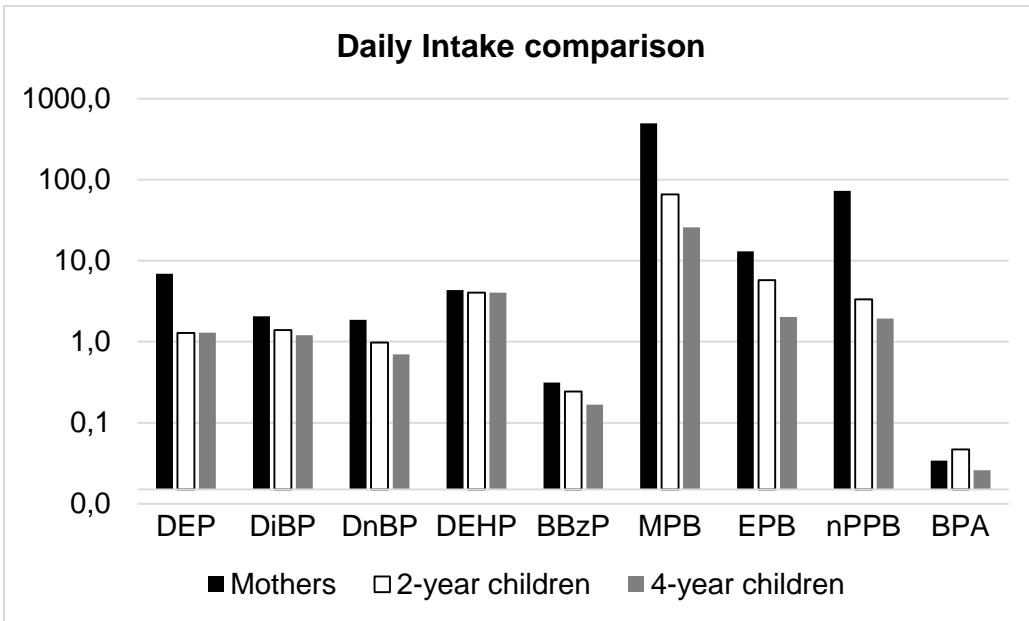**Figure S2.** Estimated daily intake comparison for Rhea cohort subjects, median values (μg d^{-1} kg^{-1}), y axis in logarithmic scale

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