Human-Biomonitoring für den alternativen Weichmacher Di(2-ethylhexyl)adipat (DEHA)

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Alexandra Gotthardt geb. Nehring (M.Sc.)

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Abkürzungsverzeichnis

5cx-MEPA	Mono(5-carboxy-2-ethylpentyl)adipat
5OH-MEHA	Mono(2-ethyl-5-hydroxyhexy)ladipat
5oxo-MEHA	Mono(2-ethyl-5-oxohexyl)adipat
Abb.	Abbildung
AA	Adipinsäure
ACN	Acetonitril
BBP	Benzylbutylphthalat
BE	Biomonitoring Äquivalente, engl. *biomonitoring equivalents*
BMU	Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit
bspw.	beispielsweise
bw	Körpergewicht, engl. *body weight*
CE	Kollisionsenergie, engl. *collision energy*
CHMS	engl. *Canadian Health Measures Survey*
c_{max}	Maximale Urinkonzentration
CoRAP	engl. *Community Rolling Action Plan*
cx-MIOA	Mono(carboxy-isooctyl)adipat
DBP	Dibutylphthalat
$ddMS^2$	Datenabhängige Tandem-Massenspektrometrie; engl. *data dependent tandem mass spectrometry*
DEHA	Di(2-ethylhexyl)adipat
DEHP	Di(2-ethylhexyl)phthalat
DEHTP	Di(2-ethylhexyl)terephthalat
DI	Tägliche Aufnahmemenge, engl. d*aily intake*
DIBP	Diisobutylphthalat
DINA	Diisononyladipat
DINCH	1,2-Cyclohexandicarbonsäurediisononylester
DINP	Diisononylphthalat
DnBA	Di-*n*-butyl-adipat
DNS	Desoxyribonukleinsäure

DOA	Dioctyladipat
DP	engl. *declustering potential*
DPHP	Di(2-propylheptyl)phthalat
e.g.	Zum Beispiel, latein. *exempli gratia*
ECHA	Europäische Chemikalienagentur
EHS	2-Ethylhexylsalicylat
ESI	Elektrospray-Ionisation
EU	Europäische Union
FCM	Lebensmittelkontaktmaterial, engl. *food contact material*
Fig.	Abbildung, engl. *figure*
F_{UE}	Konversionsfaktor, engl. *urinary excretion fraction*
GerES	Deutsche Umweltstudie zur Gesundheit, engl. *German Environmental Survey*
HBM	Human-Biomonitoring
HMW Phthalate	Hochmolekulare Phthalate, engl. *high molecular weight phthalates*
HPLC	Hochleistungsflüssigchromatographie, engl. *high performance liquid chromatography*
HRMS	Hochauflösende Massenspektrometrie, engl. *high-resolution mass spectrometry*
KG	Körpergewicht
LC	Flüssigchromatographie; engl. *liquid chromatography*
LMW Phthalate	Niedermolekular Phthalate, engl. *low molecular weight phthalates*
LOD	Nachweisgrenze, engl. *limit of detection*
LOQ	Bestimmungsgrenze, engl. *limit of quantification*
m/z	Masse-zu-Ladungs-Verhältnis
MRM	engl. *multiple reaction monitoring*
MS	Massenspektrometrie
MS/MS	Tandem-Massenspektrometrie
NHANES	engl. *National Health and Nutrition Examination Survey*
NMR	Kernspinresonanz(-spektroskopie); engl. *nuclear magnetic resonance (spetroscopy)*
NOAEL	engl. *No Observed Adverse Effect Level*
NOEL	engl. N*o Observed Effect Level*

OC	Octocrylen
OH-MINA	Mono(hydroxy-isononyl)adipat
oxo-MINA	Mono(oxo-isononyl)adipat
PVC	Polyvinylchlorid
Q	Qualitätskontrollmaterial
QqQ	Triple-Quadrupol
REACH	Registrierung, Bewertung, Zulassung und Beschränkung chemischer Stoffe, engl. *Registration, Evaluation, Authorization and Restriction of Chemicals*
RV95	Referenzwert
SCF	Wissenschaftlicher Lebensmittelausschuss, engl. *EU Scientific Committee on Food*
SIVA	Stabilisotopenverdünnungsanalyse
SML	Spezifisches Migrationslimit, engl. *specific migration limit*
SPE	Festphasenextraktion, engl. *solid phase extraction*
$t_{1/2}$	Eliminations-Halbwertszeit
TDI	Tolerierbare tägliche Aufnahmemenge, engl. *tolerable daily intake*
TFC	Flüssigchromatographie mit turbulenten Strömungen, engl. *turbulent flow chromatography*
t_{max}	Zeitpunkt der maximalen Urinkonzentration
U.S.	Vereinigten Staaten von Amerika, engl. *United States of America*
U.S. EPA	United States Environmental Protection Agency
UBA	Umweltbundesamt
VCI	Verband der chemischen Industrie e.V.
z.B.	Zum Beispiel

Inhaltsverzeichnis

Kapitel 1

Einleitung und Zielsetzung

Bei Di(2-ethylhexyladipat) (DEHA) handelt es sich um einen alternativen Weichmacher der in einer Vielzahl an Verbraucherprodukten eingesetzt wird, da DEHA als Substitut für die stark regulierten bzw. verbotenen Phthalat-Weichmacher verwendet werden kann (Stuer-Lauridsen et al. 2001; BASF 2019; Malveda et al. 2015; SPIN database). Da DEHA - genauso wie die Phthalat-Weichmacher - nicht chemisch kovalent in den Kunststoffen gebunden ist (Graham 1973), kann es aus diesen herausmigrieren und so eine Belastung von Anwendern, bzw. der allgemeinen Bevölkerung resultieren. Erste Abschätzungen der möglichen äusseren Belastungen (z.B. durch Frischhaltefolien) deuten darauf hin, dass diese Belastungen unter worst-case Annahmen durchaus toxikologisch relevant sein können (Daun and Gilbert 1977; Castle et al. 1987; Till et al. 1987; Højslev Petersen and Tubæk Naamansen 1998). Eine valide Bestimmung der inneren Exposition, d.h. die Erfassung der Gesamtheit aller Expositions- und Aufnahmewege, war bislang nicht möglich da geeignete Expositions-Biomarker nur unzulänglich beschrieben waren und hinreichend empfindliche und validierte Methoden zur Bestimmung dieser in human-biologischem Material nicht existierten. Ziel dieser Arbeit ist deshalb die Entwicklung eines validen und robusten Human-Biomonitoring-Ansatzes für DEHA, um DEHA-Belastungen von Anwendern, der Allgemeinbevölkerung oder potentiell erhöht belasteten oder besonders suszeptiblen Bevölkerungsgruppen beschreiben zu können und somit eine Grundlage für die valide Abschätzung der Gesamtbelastung und des daraus resultierenden gesundheitlichen Risikos zu schaffen. Mit diesem Ziel wurde DEHA im Jahr 2014 als eine von 50 Substanzen für ein bisher einzigartiges Kooperationsprojekt zur Förderung des HBM zwischen Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit (BMU) und dem Verband der Chemischen Industrie e. V. (VCI) ausgewählt (Kolossa-Gehring et al. 2017; UBA 2020).

Kapitel 2

Theoretische Grundlagen

Im Folgenden wird auf das Human-Biomonitoring als Instrument zur Expositions- und Risikobeurteilung im Gesundheitsschutz eingegangen sowie dessen Anwendung bei der Beschreibung der inneren Belastung gegenüber Weichmachern. Zudem wird ein Überblick über den in dieser Arbeit behandelten Weichmacher Di(2-ethylhexyl)adipat gegeben.

2.1. Human-Biomonitoring

2.1.1. Definition Human-Biomonitoring

Im täglichen Leben kommt der menschliche Organismus mit einer Vielzahl an natürlichen und anthropogenen Chemikalien in Kontakt. Durch eine orale, inhalative oder dermale Aufnahme (oftmals auch in komplexer Kombination) resultiert eine innere Belastung (Exposition) des menschlichen Organismus gegenüber diesen Chemikalien, welche zu gesundheitlichen Beeinträchtigungen führen kann. Unter Human-Biomonitoring (HBM) wird die Bestimmung der inneren Belastung gegenüber Schad-/Gefahrstoffen durch die Quantifizierung von geeigneten Biomarkern in human-biologischen Materialien (z.B. Blut oder Urin) verstanden (Komm. HBM 1996a; Ewers et al. 1999; Angerer et al. 2007; Needham et al. 2007).

Das HBM wird in zwei unterschiedliche Ansätze zur Bestimmung der inneren Belastung unterteilt. Im Rahmen des Effektmonitorings werden durch die Belastung ausgelöste gesundheitliche Effekte auf biochemischer bzw. biologischer Ebene untersucht. Als Effektbiomarker werden unter anderem DNS- oder Protein-Addukte genutzt oder direkte biologische Veränderungen auf Zellebene, wie beispielsweise Veränderungen in der Enzymaktivität. Beim Belastungsmonitoring (auch Expositions-Monitoring genannt) erfolgt die direkte Bestimmung der Konzentration von Stoffen oder deren Metaboliten in menschlichen Matrices. Die Konzentration der Expositionsbiomarker wird für die quantitative Beschreibung der inneren Belastung gegenüber der zu untersuchenden Substanz herangezogen. Die Abschätzung des gesundheitlichen Risikos, resultierend aus der inneren Belastung (ggf. extrapoliert auf die äußere Belastung), beruht auf möglichen konzentrationsabhängig auftretenden gesundheitlichen Beeinträchtigungen (Komm. HBM 1996a; Angerer et al. 2007; Angerer and Weiß 2007).

2.1.2. Anwendungsbereiche des Human-Biomonitoring

Der Vorteil des HBM besteht in der Erfassung der individuell tatsächlich aufgenommenen Dosis (innere Belastung), da alle Aufnahmepfade und Expositionsquellen kumulativ erfasst werden sowie individuelle Faktoren in der Aufnahme, Metabolismus und Exkretion des Stoffs gleichermaßen in das Untersuchungsergebnis eingehen. In vielen Fällen ist ein direkter Rückschluss auf Expositionsquellen bzw. den Beitrag verschiedener Expositionsquellen durch das HBM nicht möglich. Hierfür steht durch das Umwelt-Monitoring, auch Ambient-Monitoring genannt, ein ergänzendes Untersuchungsinstrument zur Verfügung. Durch die Messung von Stoffkonzentration in Umweltmedien wie Luft, Wasser, Boden, Lebensmitteln und anderen Konsumgütern können äußere Expositionsquellen identifiziert und deren jeweiliger Expositionsbeitrag abgeschätzt werden. Eine Abschätzung der inneren Gesamtbelastung und dem damit verbundenem gesundheitlichem Risiko durch das Umwelt-Monitoring basiert auf einer Vielzahl von bekannten und (möglicherweise) unbekannten Quellen sowie theoretisch begründete Annahmen und ist somit oftmals komplexer und fehleranfälliger als die integrale Bestimmung über das HBM. (Komm. HBM 1996a; Angerer et al. 2007; Angerer and Weiß 2007). Das HBM stellt somit ein wichtiges Instrument in der Expositions- und Risikoabschätzung dar, welches auf individueller Ebene oder auf die Belastungssituation von ganzen Bevölkerungsgruppen angewandt werden kann. Darüber hinaus können durch HBM-Untersuchungen zeitliche Veränderungen in der Belastung erkannt und Entwicklungstrends (einphasen und ausphasen bestimmter Chemikalien, Substitutionsprozesse) nachvollzogen werden (Komm. HBM 1996a). Dies wird insbesondere zur Überprüfung der Effektivität von getroffenen Schutzmaßnahmen im Rahmen des europäischen Chemikalienrechts REACH (Registrierung, Bewertung, Zulassung und Beschränkung chemischer Stoffe, engl. *Registration, Evaluation, Authorization and Restriction of Chemicals*) genutzt (Boogaard et al. 2011; Boogaard et al. 2012; Apel et al. 2017; Ganzleben et al. 2017). HBM wird in der Arbeits- und Umweltmedizin sowie in der nationalen wie internationalen Gesundheitspolitik zunehmend als effektives und objektives Werkzeug zur Expositions- und Risikobeurteilung und damit zum effektiven Gesundheitsschutz eingesetzt (Komm. HBM 1996a; Angerer et al. 2007; Kolossa-Gehring 2012a; Ganzleben et al. 2017; Joas et al. 2017).

2.1.3. Human-biologische Untersuchungsmaterialien

Als bevorzugtes human-biologisches Untersuchungsmaterial werden für das Human-Biomonitoring Blut und Urin herangezogen, da diese leicht zugänglich und in ausreichender Menge verfügbar sind. Untersuchungen in Haaren, Nägeln, Humanmilch, Atemkondensat oder menschlichem Gewebe spielen (aus verschiedensten, hier nicht näher erläuterten Gründen) eine eher untergeordnete Rolle. In großangelegten Bevölkerungsstudien wird Urin als Untersuchungsmaterial bevorzugt, da es nicht-invasiv und ohne kostenintensives medizinisches Personalgewonnen werden kann (Angerer et al. 2007; Smolders et al. 2009). Die Auswahl der geeigneten biologischen Matrix erfolgt jedoch vor allem nach der physikochemischen Eigenschaft des zu untersuchenden Stoffs sowie dessen Toxikokinetik. Bedingt durch die lange biologische Halbwertszeit und die geringe Ausscheidungsrate über den Urin werden persistente, bioakkumulative Stoffe vorwiegend im Blut nachgewiesen. Nicht-persistente Stoffe werden hingegen durch eine schnelle Ausscheidung über den Urin charakterisiert. Die dadurch resultierende kürzere Nachweisdauer im Blut sowie die gewöhnlich geringere Stoffkonzentrationen im Blut, führen zu einer bevorzugten Untersuchung nicht-persistenter Stoffe im Urin. (Needham and Sexton 2000; Needham et al. 2007; Koch and Calafat 2009)

Für eine erleichterte Ausscheidung über den Urin können Substanzen über den Stoffwechsel funktionalisiert werden. So entstehen beispielsweise oxidativ modifizierte Metaboliten (Phase I Metabolismus). Für eine weitere Erhöhung der Wasserlöslichkeit, können die Fremdstoffe selbst oder ihre funktionalisierten Metaboliten zudem an stark wasserlösliche Stoffe gebunden (konjugiert) werden, wie beispielsweise Glucuronsäure oder Sulfat (Phase II Metabolismus). Je nach Substanz können so mit verschiedenen Geschwindigkeiten eine Vielzahl unterschiedlich polarer Phase I und Phase II Metaboliten gebildet werden, welche sich durch unterschiedliche physikochemischen Eigenschaften auszeichnen. Die über den Urin ausgeschieden Metaboliten können dann als Expositionsbiomarker im HBM herangezogen werden. Zur Vereinfachung der Interpretation und Analytik werden oftmals die Phase II Metaboliten (Konjugate) durch Hydrolyse in die entsprechenden Phase I Metaboliten zurückgeführt (Koch and Calafat 2009; Calafat et al. 2015a). Die im Urin ermittelte absolute Konzentration der Expositionsbiomarker ist neben der stoffspezifischen Toxikokinetik auch von der Harnproduktion der Nieren (Diurese) abhängig, welche jedoch u.a. abhängig vom individuellen Wasserhaushalt oder körperlicher Anstrengung, Schwankungen unterliegen kann. Die daraus resultierende variable Verdünnung des Urins kann durch einen Korrekturfaktor ausgeglichen werden,

um so die individuelle innere Belastung vergleichbarer zu bewerten. In den meisten HBM-Studien wird der Kreatiningehalt einer Urinprobe als diuresebedingte Bezugsgröße herangezogen. Bei Kreatinin handelt es sich um ein Stoffwechselprodukt, welches weitestgehend konstant von der Niere an den Urin abgegeben wird. Die Normierung der gemessenen Biomarkerkonzentration im Urin auf den Kreatiningehalt der Urinprobe ermöglicht eine Expositionsbewertung weitestgehend unabhängig von diuresebedingten Schwankungen. Da die individuelle Kreatininproduktion auch abhängig von Alter, Geschlecht, Gewicht und Muskelmasse ist, wird alternativ in einigen Studien die Urindichte oder die Osmolarität als Korrekturfaktor herangezogen (Alessio et al. 1985; Greenberg and Levine 1989; Boeniger et al. 1993; Bundesgesundheitsblatt 2005; Barr et al. 2005; Cone et al. 2009; Gerchman et al. 2009; Cocker et al. 2011; Smolders et al. 2009; Lorber et al. 2011; Aylward et al. 2014). In 24-Stunden Urinproben, wie z.B gesammelt in der Umweltprobenbank (Kolossa-Gehring et al. 2012b), entfällt jedoch diese Notwendigkeit zur Normierung und tägliche Belastungen (daily intakes) lassen sich schnell und robust ableiten (siehe 2.1.4.).

2.1.4. Interpretation von HBM-Daten

In HBM-Studien erfolgt die Messung von Expositionsbiomarkern in menschlichen Körperflüssigkeiten zur gezielten Erfassung der inneren Exposition. Für eine einheitliche wissenschaftliche Interpretation und Bewertung von Messdaten hinsichtlich der inneren Belastungssituation haben sich national und international Referenz- und Beurteilungswerte etabliert (Ewers et al. 1999; Komm. HBM 1996b, 2014; Angerer et al. 2011).

Der Referenzwert (RV95) entspricht der Angabe des 95%-Konfidenzintervalls des 95. Perzentils der absolut gemessenen Stoffkonzentration in einer Referenzbevölkerung. Es handelt sich um eine rein statistische Auswertung der Daten, die eine Beschreibung der Hintergrundexposition der Referenzbevölkerung zum Zeitpunkt der Probennahme ermöglicht. Im Rahmen der individuellen Expositionsbewertung kann der Referenzwert eines Stoffs als Bezugs- bzw. Vergleichswert genutzt werden. Eine Überschreitung des RV95 zeigt so eine über die „normale“ Hintergrundbelastung hinausgehende Belastung an (bspw. durch zusätzliche individuelle Belastungspfade in der Umwelt oder Arbeit). Bedingt durch zeitliche Veränderungen der Belastungssituation über Jahre bzw. Jahrzehnte ist es sinnvoll durch regelmäßig durchgeführte Populationsstudien den Referenzwert zu aktualisieren, um

so einen Vergleich zur aktuellen Belastungssituation zu ermöglichen. Dabei ist hervorzuheben, dass durch den Referenzwert (bzw. dessen Überschreitung) eine Beurteilung des individuellen Gesundheitsrisikos grundsätzlich nicht möglich ist, da es sich um keinen toxikologisch abgeleiteten Wert handelt (Ewers et al. 1999; Komm. HBM 1996b, 2014; Angerer et al. 2011; Apel et al. 2017).

Im Rahmen der Risikobewertung erfolgt eine Abschätzung möglicher gesundheitlicher Beeinträchtigung, beruhend auf den Messdaten aus HBM-Studien und dem konzentrationsabhängigen Auftreten toxikologischer bzw. gesundheitsadverser Effekte (Wirkungsschwelle) (Komm. HBM 1996b; Needham et al. 2007; Angerer et al. 2011; Joas et al. 2017). Verschiedene Ansätze haben sich hierfür etabliert. Basierend auf der gemessenen Konzentration des Expositionsbiomarkers im untersuchenden Medium kann eine Abschätzung (Rückrechnung) der täglichen Aufnahmemenge (DI, engl. *daily intake*) pro Kilogramm Körpergewicht erfolgen. Die Bewertung potentieller Gesundheitsrisiken erfolgt dann über toxikologisch abgeleitete Grenzwerte in gleicher Einheit/Größenordnung, wie z.B. die Tolarable Tägliche Aufnahmemenge (TDI, tolerable daily intake) oder die Akzeptable Tägliche Aufnahmemenge (ADI, acceptable daily intake) (Kohn et al. 2000; David 2000; Angerer et al. 2011; Aylward et al. 2012). Als weiteres Konzept wurden Human-Biomonitoring-Werte (HBM-I- und HBM-II-Wert) als gesundheitsbezogene Beurteilungswerte von der „Kommission Human-Biomonitoring“ etabliert. Basierend auf epidemiologischen oder toxikologischen Studien werden Konzentrationen eines oder mehrerer Expositionsbiomarker im Blut oder Urin als toxikologisch begründete Beurteilungswerte für die innere Belastung abgeleitet. Stark vereinfacht entspricht der HBM-I-Wert der Biomarker-Konzentration (in Blut oder Urin), die bei kontinuierlicher Exposition am TDI bzw. ADI resultieren würde (Komm. HBM 1996b, 2014; Ewers et al. 1999; Angerer et al. 2011; Apel et al. 2017). Eine Unterschreitung des HBM-I-Werts führt somit nach aktuellem Kenntnisstand zu keinen gesundheitlichen Beeinträchtigungen, sodass kein Handlungsbedarf besteht. Bei Überschreitung des HBM-I-Wertes kann eine gesundheitliche Beeinträchtigung nicht mehr sicher ausgeschlossen werden. Eine Überprüfung des zeitlichen Verlaufs zusammen mit einer Verminderung der Exposition unter hinnehmbaren Aufwand wird empfohlen. Bei einer Überschreitung des HBM-II-Wertes hingegen besteht akuter Handlungsbedarf für Maßnahmen zur Minimierung der Exposition, da gesundheitliche Beeinträchtigungen als möglich angesehen werden (Komm. HBM 1996b). HBM-Werte werden in der Regel bezogen auf eine lebenslange Belastung abgeleitet (Apel et al. 2017). Weitere toxikologisch abgeleitetete Beurteilungswerte, ähnlich den HBM-Werten, sind die sogenannten Biomonitoring-Äquivalente (BE, engl. *biomonitoring equivalents*), welche die gesundheitliche Relevanz einer Belastung ebenfalls durch

die Konzentrationsangabe von Biomarkern im entsprechenden Körpermedium charakterisieren (Hays et al. 2007; Angerer et al. 2011; Hays and Aylward 2012). Ein zusammenfassender Vergleich der Ableitungsbedingungen von HBM- und BE-Werten befindet sich in Angerer et al. (2011).

Verfahren zur Ableitung gesundheitsbezogener Beurteilungswerte (wie HBM-Werte) basieren im Idealfall auf der Charakterisierung der Wirkungsschwellen der Chemikalien. Humandaten aus epidemiologischen Studien sind jedoch nur für wenige Stoffe verfügbar sodass Ableitungsverfahren auch auf Basis von Tierstudien etabliert wurden (Komm. HBM 2007a, 2007b, 2014). Um einen toxikologischen Grenzwert (abgeleitet aus Human- oder Tierstudien) in eine äquivalente Biomarkerkonzentration umzurechnen, muss der Zusammenhang zwischen der Biomarkerkonzentration im human-biologischen Medium und der externen Exposition gegenüber der Ausgangssubstanz untersucht werden. Ohne dieses Wissen kann weder eine Expositionsbeurteilung noch eine Risikobeurteilung stattfinden (Koch and Calafat 2009; Angerer et al. 2011; Komm. HBM 2014; Apel et al. 2017). Informationen über die Verstoffwechselung der Ausgangssubstanz und die Eliminierung aus dem menschlichen Organismus (z.B. Ausscheidung polarer Metaboliten über den Urin) bilden hierfür die Grundlage. Im Rahmen von humanen Toxikokinetikstudien werden Konversionsfaktoren (F_{UE}, engl. *urinary excretion fraction*) abgeleitet, welche den Dosisanteil beschreiben, der als Expositionsbiomarker über den Urin ausgeschieden wird. Dies ermöglicht die Umrechnung von toxikologischen Grenzwerten (wie dem TDI) zu äquivalenten Biomarkerkonzentrationen sowie die präzise Rückrechnung auf die individuelle aufgenommene tägliche Dosis, basierend auf der gemessenen Biomarkerkonzentration im Urin (Wittassek et al. 2011; Angerer et al. 2011). Infolgedessen ermöglichen humane Toxikokinetikstudien eine Beurteilung der Biomarkerkonzentration hinsichtlich der inneren Belastungssituation, aber auch der sie bedingenden äußeren Gesamtbelastung (aufgenommene Dosis) und bilden zusammen mit toxikologischen Human- oder Tierstudien ein Bewertungsinstrument für die gesundheitliche Risikoabschätzung (Angerer et al. 2011; Wittassek et al. 2011; Komm. HBM 2014; Apel et al. 2017).

2.2. Weichmacher

2.2.1. Definition Weichmacher

Als Weichmacher werden chemische Additive bezeichnet, welche Kunststoffen zugesetzt werden, um spezielle Produkteigenschaften zu erzielen. Beispielsweise wird durch den Zusatz von Weichmachern bei ursprünglich hartem, sprödem und brüchigem Polyvinylchlorid (PVC) die Dehnbarkeit, Flexibilität und Elastizität des Kunststoffs erhöht. Durch die modifizierten Eigenschaften des PVC werden weitere Anwendungen wie in Folien, Kabelisolierungen oder in Spielzeug möglich (Graham 1973; Rahman and Brazel 2004; Plastics Europe 2006; Malveda et al. 2015). Weichmacher gehören zu Industriechemikalien mit einem hohem Produktionsvolumen weltweit. Im Jahr 2014 wurden mehr als acht Millionen Tonnen Weichmacher weltweit produziert (Malveda et al. 2015).

2.2.2. Phthalate

Die am häufigsten verwendeten Weichmacher sind Phthalate (Malveda et al. 2015). Als Phthalate werden Dialkylester der Phthalsäure (1,2-Benzoldicarbonsäure) bezeichnet (**Abb. 1**). Je nach Länge der enthaltenen Alkylkette unterscheiden sich die toxikologischen Profile sowie die Anwendungsbereiche. Hochmolekulare Phthalate (HMW phthalates, engl. *high molecular weight phthalates*) werden vorwiegend als Weichmacher in PVC eingesetzt, während niedermolekulare Phthalate (LMW phthalates, engl. *low molecular weight phthalates*) primär als Lösungsmittel und Formulierungshilfen in anderen Anwendungen als im Kunststoffsektor verwendet werden (Duty et al. 2005; Wittassek et al. 2011; Koch et al. 2013a; Parlett et al. 2013; Malveda et al. 2015; Ackerman et al. 2014; BAG Schweiz 2019). Das am häufigsten eingesetzte Phthalat war lange Zeit Di(2-ethylhexyl)phthalat (DEHP) (Malveda et al. 2015), bis dessen Anwendung in Europa zunehmend gesetzlich reguliert und seit 2015 mit nur wenigen Ausnahmen verboten worden ist (European Commission 2011b).

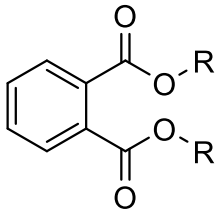

Abb. 1: *Chemische Struktur Phthalate*

Zusammen mit weiteren Phthalaten wurde bei DEHP in Tierstudien ein negativer Einfluss auf die männliche Fruchtbarkeit bei pränataler Exposition (Exposition des Nachkommens im Uterus) nachgewiesen. Die durch Phthalate hervorgerufenen Effekte in männlichen Nachkommen werden als „Phthalat-Syndrom“ bezeichnet und kennzeichnen sich unter anderem durch eine verringerte Spermienzahl, Unfruchtbarkeit und Missbildungen der Fortpflanzungsorgane. Die beschriebenen toxikologischen Wirkungen können auf antiandrogene Effekte (Störung des männlichen Sexualhormonhaushalts) der Phthalate zurückgeführt werden (Gray et al. 2000; Mylchreest et al. 2000; Parks et al. 2000; Foster et al. 2001; Kavlock et al. 2002; Lee et al. 2004; Jarfelt et al. 2005; Foster 2006; Borch et al. 2006; Saillenfait et al. 2006; Wilson et al. 2008; Lyche et al. 2009; Martino-Andrade and Chahoud 2010; Hannas et al. 2011; Kay et al. 2014; Koch et al. 2015; Koch 2016; Dobrzyńska 2016; Rowdhwal and Chen 2018; BAG Schweiz 2019). Aufgrund des toxikologischen Wirkungsprofils wurden Phthalate, welche drei bis sieben Kohlenstoffatome in ihrer längsten Alkylkette enthalten und eine Gesamtkohlenstoffanzahl von acht im verzweigten bzw. unverzweigten Alkylrest nicht übersteigen, als reproduktionstoxisch und endokrine Disruptoren (Chemikalien, die den Hormonhaushalt beeinflussen) eingestuft sowie in die Liste der besonders besorgniserregenden Stoffe aufgenommen (Komm. EG 2004; EU Parlament 2006, 2008; Koch et al. 2015; Koch 2016; EU-Kommission 2017; ECHA 2021a, 2021b, 2021c, 2021d).

In Europa wurden erste Einsatzbeschränkungen für bestimmte Phthalate, darunter DEHP, bereits im Jahr 1999 für Kinderspielzeug und Babyartikel erlassen (European Commission 1999). In nachfolgenden Jahren erfolgte eine zunehmend umfassendere gesetzliche Regulierung auf europäischer Ebene mit weiteren Anwendungsbeschränkungen und -verboten (European Parliament 2005, 2009; European Commission 2011a). Auch im 2006 erneuerten europäischen Chemikalienrecht wurden rechtlich bindende Regulierungen für die Verwendung von Phthalaten im Rahmen der REACH-Verordnung umgesetzt (European Parliament 2006) und über die letzten Jahre ausgeweitet. Seit 2015

unterliegen DEHP und drei weitere Phthalate (Diisobutylphthalat (DIBP), Dibutylphthalat (DBP), Benzylbutylphthalat (BBP)) einer Zulassungspflicht (European Commission 2011b), welche seit 2018 auch für den Import gilt (European Commission 2018). Diese Zulassungspflicht bedeutet faktisch ein Verwendungsverbot, außer in speziellen und nur in Ausnahmefällen autorisierten Anwendungen. In Kanada (Canada 2010, 2016) und den Vereinigten Staaten von Amerika (CPSC 2008) gelten ebenfalls gesetzliche Regulierungen für die Anwendung von Phthalaten. Durch den weiterhin hoch gebliebenen weltweiten Bedarf an Weichmachern, dem Ziel das Inverkehrbringen von Waren zu erleichtern sowie sichere Produkte für den Verbraucher herzustellen, befindet sich der Weichmachermarkt in einem Substitutionsprozess von Phthalaten hin zu alternativen Weichmachern, mit den Adipaten als eine mögliche Alternative (Rahman and Brazel 2004; Calafat et al. 2015b; Bui et al. 2016; Koch 2016; BAG Schweiz 2019).

2.2.3. Human-Biomonitoring von Weichmachern

Die Mehrheit der verwendeten Weichmacher sind chemisch nicht kovalent mit den Kunststoffpolymeren verbunden, sondern zwischen den Polymerketten eingelagert. Dadurch können sich die Moleküle aus dem Kunststoff herauslösen und in angrenzende Medien (z.B. Wasser, Fett oder Lebensmittel) übertreten (Graham 1973; Rahman and Brazel 2004). Zusammen mit der vielseitigen Anwendung von Phthalaten führt dies zu einer nachweisbaren ubiquitären Verbreitung von Phthalaten in der Umwelt und einer resultierenden inneren Belastung in der Bevölkerung (Koch et al. 2003a; Koch et al. 2003c; Horn et al. 2004; Wormuth et al. 2006; Fromme et al. 2007; Abb et al. 2009; Wittassek et al. 2011; Fromme et al. 2013; Koch et al. 2013a; Parlett et al. 2013; Ackerman et al. 2014; Bertoncello Souza et al. 2018). Die Exposition gegenüber Phthalaten kann durch HBM-Daten charakterisiert werden sowie der zeitliche Verlauf und die damit verbundene Effektivität der gesetzlichen Regulation zur Reduzierung des gesundheitlichen Risikos durch Phthalate überprüft werden (Wittassek et al. 2011; Calafat et al. 2015b; Koch et al. 2017; BAG Schweiz 2019; Apel et al. 2020). Für eine Vielzahl an Phthalaten wurden Expositionsbiomarker identifiziert, HBM-Methoden etabliert und humane Metabolismusstudien bzw. Toxikokinetikstudien durchgeführt, sodass eine breite wissenschaftliche Basis für das HBM von Phthalaten geschaffen wurde (Schmid and Schlatter 1985; Koch et al. 2003b; Koch et al. 2003c; Koch et al. 2004; Kato et al. 2005; Preuss et al. 2005; Silva et al. 2004; Koch et al. 2005; Koch and Angerer 2007; Koch et al. 2007; Anderson et al. 2011; Wittassek et al. 2011; Koch et al. 2012; Kessler et al. 2012; Koch 2016; Klein et al. 2018; Wang et al. 2019). Mit dem Substitutionsprozess von Phthalaten hin zu Ersatzprodukten wuchs zunehmend der Bedarf an HBM-Methoden für alternative Weichmacher und erste HBM-Methoden für die nicht-phthalatbasierten Weichmacher 1,2-Cyclohexandicarbonsäurediisononylester (DINCH) (Schütze et al. 2012) und Di(2-ethylhexyl)terephthalat (DEHTP) (Lessmann et al. 2016a) wurden bereits in 2012 bzw. 2016 publiziert.

In HBM-Bevölkerungsstudien aus Deutschland, USA und Kanada konnte ein rückläufiger Trend der Belastung gegenüber diesen stark regulierten (bzw. inzwischen verbotenen) Phthalate nachvollzogen werden, während in den Belastungen gegenüber alternativen Weichmachern ein Anstieg aufgezeigt werden konnte (Wittassek et al. 2007; Göen et al. 2011; Silva et al. 2013a; Zota et al. 2014; Schütze et al. 2014; Schütze et al. 2015; Calafat et al. 2015b; Koch et al. 2017; Haines et al. 2017; Kasper-Sonnenberg et al. 2019; Silva et al. 2019; Lessmann et al. 2019; Schmidtkunz et al. 2019; Apel et al. 2020; Frederiksen et al. 2020).

2.3. Di(2-ethylhexyl)adipat (DEHA)

2.3.1. Allgemeine Informationen

Neben den bereits genannten Weichmachern DINCH und DEHTP wird auch Di(2-ethylhexyladipat) (DEHA) als eines der relevantesten Phthalat-Alternativen/Substitute erachtet. DEHA gehört zur Gruppe der Adipate, wie die Dialkylester der Adipinsäure (1,6-Hexandicarbonsäure) allgemein bezeichnet werden (**Abb. 2 und 3).** DEHA ist mengenmäßig das am häufigsten eingesetzte Adipat und ist ein Weichmacher, der sich vor allem wegen seiner Kältebeständigkeit auszeichnet. (Graham 1973; Stuer-Lauridsen et al. 2001; BASF 2019; Malveda et al. 2015)

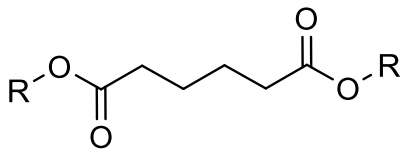

Abb. 2: *Allgemeine chemische Struktur der Adipate*

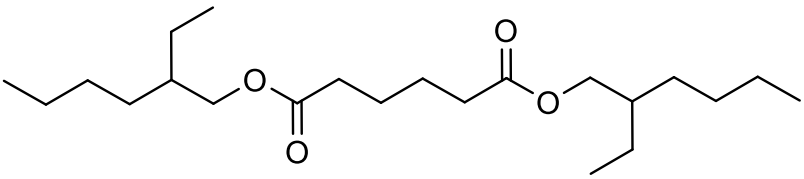

Abb. 3: *Chemische Struktur DEHA*

2.3.2. Anwendungsbereiche von DEHA

Für DEHA existieren keine, zu den Phthalaten insbesondere DEHP vergleichbaren, Anwendungsbeschränkungen oder -verbote. Der Anwendungsbereich von DEHA ist vielseitig und reicht von Verbraucherprodukten aus Weich-PVC (u.a. Kinderspielzeug), bis hin zu industriellen Anwendungen. Dort wird DEHA beispielsweise in Bodenbelägen und Wandverkleidungen, Wasch- und Reinigungsprodukten, Schmiermitteln, Klebstoffen oder kältefesten Farben und Lacke verwendet. Des Weiteren wird DEHA in medizinischen Geräten wie in Dialyseschläuchen eingesetzt (Graham 1973; Stuer-Lauridsen et al. 2001; Rahman and Brazel 2004; Biedermann-Brem et al. 2008; BASF 2019;

Gimeno et al. 2014; Bernard et al. 2014; Malveda et al. 2015; Malarvannan et al. 2019; SPIN database; ECHA 2021e) und ist in Europa und weiteren Teilen der Welt (bspw. USA, Kanada und China) ebenfalls in Lebensmittelkontaktmaterialien (z.B. Frischhaltefolie) zugelassen (EFSA 2008; European Commission 2011a; FDA 2019; Canada 2020; NHFPC China 2016; European Commission 2019).

2.3.3. Toxikologisches Profil von DEHA

Für DEHA existieren, von allen unter REACH zugelassenen Adipaten, die am meisten durchgeführten toxikologischen Studien und die größte toxikologische Datenbasis im Rahmen der Erfassung und Bewertung der Substanzgefahren unter REACH. Durchgeführte Toxizitätsstudien mit DEHA bilden dabei häufig die Basis für ein *read-across-category* Verfahren bei anderen Adipaten wie Di-*n*-butyl-adipat (DnBA) oder Diisononyladipat (DINA) (ECHA 2021h, 2021f, 2021i). Im Vergleich zu DEHP kennzeichnet sich das toxikologische Profil von DEHA bislang durch ein deutlich geringeres gesundheitliches Gefährdungspotential. Eine vergleichbare Wirkung auf die Fortpflanzungsfähigkeit männlicher Nachkommen nach pränataler Exposition wie bei DEHP konnte nicht beobachtet werden (Dalgaard et al. 2003; Kang et al. 2006; Nabae et al. 2006; Miyata et al. 2006). Verschiedene Studien beschreiben jedoch reproduktionstoxische Effekte von DEHA in Ratten bei vergleichbar hohen Verabreichungsmengen (Dalgaard et al. 2003; Miyata et al. 2006; Wato et al. 2009), für eine abschließende Substanzbewertung hinsichtlich des möglichen reproduktionstoxischen Potentials wurde DEHA in den Community Rolling Action Plan (CoRAP) der ECHA inkludiert (Ergebnis noch ausstehend) (ECHA 2021g). Für die derzeitige toxikologische Beurteilung von DEHA wird als Grenzwert die tolerierbare tägliche Aufnahmemenge (TDI, engl. *tolerable daily intake*) herangezogen. Für DEHA wurde ein TDI von 0,3 mg/kg KG/Tag, basierend auf beobachteten foetotoxischen Effekten in einer Teratogenitätsstudie, abgeleitet (Scientific Committee on Food 1997). Zum Vergleich, dieser Wert liegt um den Faktor 6 über dem TDI von DEHP (AFC 2005).

2.3.4. Kenntnisstand zum Human-Biomonitoring von DEHA zu Studienanfang

Vor dem Beginn dieser Promotionsstudie wurden für DEHA bisher weder spezifische Metaboliten als Expositionsbiomarker etabliert, noch ein valides HBM zur Expositionsbeurteilung durchgeführt. Wissenschaftliche Daten über den Humanmetabolismus von DEHA waren sehr begrenzt. Eine 1981 durchgeführte Metabolismusstudie an Ratten identifizierte die Ausscheidung in Form von Kohlenstoffdioxid über den Atem und als Adipinsäure (AA) über den Urin als Hauptausscheidungswege nach oraler DEHA-Aufnahme (Takahashi et al. 1981). Eine schnelle Metabolisierung von DEHA hin zu AA als Hauptmetabolit, konnte in *in vitro* Versuchen mit menschlichen Lebermikrosomen bestätigt werden (Silva et al. 2013b). AA ist jedoch kein spezifisches Stoffwechselprodukt von DEHA, da Substanzen, welche ebenfalls Adipinsäure in ihrer Struktur enthalten, wie beispielsweise andere Adipate (Bsp. DnBA), diese ebenfalls bilden können. Darüber hinaus sind exogene Quellen (AA ist in Europa und den USA als Lebenmittelzusatzstoff zugelassen) (European Commission 2012; FDA 2018) und eine mögliche endogene Bildung von AA bekannt (Pettersen et al. 1972; Liebich et al. 1980). In einer durchgeführten Humanmetabolismusstudie mit sechs Probanden wurden nach oraler Dosierung von DEHA zudem von der 2-Ethylhexansäure abgeleitete Metaboliten nachgewiesen (Loftus et al. 1993; Loftus et al. 1994). Bei den nachgewiesenen Stoffwechselprodukten im menschlichen Urin handelt es sich ebenfalls um unspezifische Metaboliten, welche von jedem Fremdstoff, welcher eine 2-Ethylhexylseitenkette enthält (wie DEHP oder DEHTP), gebildet werden können. Zudem wurden tentativ zwei spezifische Metaboliten von DEHA, mit oxidativen Modifikationen in der 2-Ethylhexylseitenkette *in vitro* mit menschlichen Lebermikrosomen und menschlichen Urinproben nachgewiesen: Mono(2-ethylhydroxyhexy)adipat (OH-MEHA) und Mono(2-ethyloxohexyl)adipat (oxo-MEHA) (Silva et al. 2013b).

2.3.5 Bisherige, externe Expositionsabschätzungen von DEHA

Eine innere Belastung der Bevölkerung gegenüber DEHA kann durch eine Vielzahl an unterschiedlichen Expositionsquellen und Aufnahmewege resultieren, da DEHA ebenfalls zu den Weichmachern gehört, die chemisch nicht kovalent mit dem Kunststoffpolymer verbunden sind sodass DEHA aus dem Kunststoff hinausmigrieren kann (Graham 1973). So ist insbesondere die Nahrungsaufnahme von verpackten Lebensmitteln als Expositionsquelle durch die direkte Aufnahme von DEHA zu erachten. Durch die Migration von DEHA aus dem Lebensmittelkontaktmaterial in das Lebensmittel kann der anschließende Verzehr zu einer direkten Aufnahme von DEHA führen. Für DEHA wurde ein spezifisches Migrationslimit (SML, engl. *specific migration limit*) von 18 mg/kg für den Einsatz in Lebensmittelkontaktmaterial festgesetzt (European Commission 2000). Es wurde jedoch bereits in mehreren Studien aufgezeigt, dass dieses SML in Abhängigkeit von der Temperatur, Lagerdauer und dem Fettgehalt des verpackten Lebensmittels durchaus deutlich überschritten werden kann (Daun and Gilbert 1977; Castle et al. 1987; Till et al. 1987; Højslev Petersen and Tubæk Naamansen 1998). Darüber hinaus stellen DEHA-enthaltende Verbraucherprodukte, Umweltmedien (wie Flüsse und Abwasser) (Horn et al. 2004; Barnabé et al. 2008), Innenraumluft und Hausstaub (Fromme et al. 2016; Christia et al. 2019; Giovanoulis et al. 2019; Hammel et al. 2019) weitere mögliche Expositionsquellen dar. Bisherige Abschätzungen der inneren Belastung/Exposition gegenüber DEHA basieren vorwiegend auf Ambient-Monitoring-Daten. Basierend auf der gemessenen Stoffkonzentration von DEHA in Lebensmittelproben (Tsumura et al. 2001; Tsumura et al. 2003; Fromme et al. 2007) oder in der Innenraumluft (Fromme et al. 2016; Subedi et al. 2017) wurden tägliche Aufnahmemengen (DI) von DEHA, unabhängig der Gesamtheit der Expositionsquellen und Aufnahmepfade sowie individuellen Faktoren, berechnet.

Kapitel 3

Durchführung

Die Entwicklung eines neuen HBM-Ansatzes für eine bislang dem HBM nicht zugängliche Substanz ist ein iterativer Prozess aus mehreren Teilschritten bzw. Teilkomponenten: 1) Auswahl theoretisch wahrscheinlicher bzw. geeigneter Metaboliten als Expositions-Biomarker. 2) Entwicklung eines analytischen Nachweisverfahrens zur quantitativen Bestimmung dieser Biomarker in human-biologischer Matrix. 3) Untersuchung dieser Biomarker in einer humanen Toxikokinetikstudie zur Bestimmung der qualitativen und quantitativen Relevanz sowie Ableitung wichtiger Kenngrößen (z.B. Kinetik, Konversionsfaktoren). 4) Überprüfung der Eignung des analytischen Verfahrens und der ausgewählten Biomarker in Bevölkerungsproben, bzw. Proben bekanntermaßen exponierter Individuen. In diesem interativen Prozess können zuvor ausgwählte Biomarker sich als nicht geeignet herausstellen oder zuvor nicht in Betracht gezogene Metaboliten als zweckmäßige Biomarker identifiziert werden. Weiterhin können Methodenoptimierungen zur Steigerung der Sensitivität der Nachweismethode oder ggf. gänzlich neue Ansätze in den vorangegangenen Schritten für die erfolgreiche Entwicklung eines HBM-Ansatzes erforderlich sein.

(Kapitel 4: Nehring et al. 2019) Die Entwicklung eines DEHA-HBM-Ansatzes erfolgte vorrangig basierend auf Kenntnissen aus bereits etablierten HBM-Ansätzen strukturähnlicher Weichmacher wie DEHP, DINCH und DEHTP. Für Weichmacher, die wie DEHA eine 2-Ethylhexyl-Alkylkette enthalten, wurden spezifische Metaboliten mit oxidativen Modifikationen (Hydroxyl-, Carbonyl- und Carboxylgruppe) in der Seitenkette erfolgreich als Biomarker im Urin etabliert (Koch et al. 2005; Anderson et al. 2011; Koch et al. 2013b; Lessmann et al. 2016b), sodass für DEHA analoge sekundär oxidierte Metaboliten als vielversprechende Expositions-Biomarker ausgewählt wurden. Für die quantitative Bestimmung der frei ausgeschiedenen und glucuronidierten DEHA-Metaboliten im Urin wurde eine analytische Methode mit online-Festphasenextraktion und anschließender Hochleistungsflüssigchromatographie gekoppelt mit Tandem-Massenspektrometrie (online-SPE-HPLC-MS/MS) entwickelt. Die Quantifizierung über Stabilisotopenverdünnungsanalyse (SIVA) mit $^{13}C_6$-markierten strukturanalogen Verbindungen wurde durch eine erstmalig durchgeführte Standard-Neusynthese (Auftragssynthese) realisiert. Um die ausreichende Sensitivität der entwickelten Methode und die Anwendbarkeit der spezifischen Metaboliten als Expositionsbiomarker zu demonstrieren, wurden erste Pilotpopulationen hinsichtlich der Belastung gegenüber DEHA untersucht. Hierdurch wurde gleichermaßen erstmals ein Einblick in die Belastungssituation von zwei verschiedenen Pilotpopulationen ohne bekannte Belastung gegenüber DEHA gegeben sowie der Beitrag der Nahrungsaufnahme als DEHA-Expositionsquelle untersucht.

(Kapitel 5: Nehring et al. 2020) Um eine tatsächliche Interpretation der gemessene Biomarkerkonzentration als Bezugsgröße für die Belastung gegenüber DEHA zu ermöglichen sowie die Anwendbarkeit der spezifischen Metaboliten als Expositions-Biomaker weiter zu überprüfen, wurde eine humanen Toxikokinetikstudie durchgeführt. Die zuvor postulierten spezifischen Metaboliten wurden hierbei erfolgreich als die am geeignetsten Biomarker identifiziert. Die im Rahmen der Kinetikstudie abgeleiteten Konversionsfaktoren (F_{UE}) ermöglichen erstmals valide Berechnungen der tatsächlich aufgenommenen Menge an DEHA (Daily Intake) unter Berücksichtigung aller Expositions- und Aufnahmewege. Angewandt auf die in Kapitel 4 beschriebenen Pilotpopulationen erfolgte darüber hinaus erstmalig eine Abschätzung potentieller Gesundheitsrisiken verschiedener (Pilot-) Bevölkerungsgruppen anhand des TDI von DEHA.

(Kapitel 6: Gotthardt et al. 2021) DINA ist ein weiteres Adipat, für welches im Rahmen des BMU/VCI-Kooperationsprojektes ein neuer HBM-Ansatz entwickelt werden sollte. Im Vergleich zu DEHA, handelt es sich bei DINA strukturell um eine isomere Alkylkette mit einem zusätzlichen Kohlenstoffatom in der Seitenkette. Wie DEHA, handelt es sich um einen alternativen Weichmacher für Phthalate mit Anwendungsbeschränkungen oder – verboten, welcher vor allem in Verbraucherprodukten eingesetzt wird. DINA ist ebenfalls nicht chemisch kovalent an das Kunststoffpolymer gebunden, wodurch von einer Belastung der Allgemeinbevölkerung ausgegangen werden kann. Im Rahmen der erfolgreichen Entwicklung eines HBM-Ansatzes für DINA wurden erstmalig Expositionsbiomarker für DINA identifiziert und Kinetikdaten für diese Biomarker inklusive Konversionsfaktoren (F_{UE}), abgeleitet. Weiterhin konnten Analogien sowie Unterschiede im Human-Metabolismus der beiden strukturell ähnlichen Adipate DINA und DEHA identifiziert werden.

Kapitel 4

Determination of human urinary metabolites of the plasticizer di(2-ethylhexyl) adipate (DEHA) by online-SPE-HPLC-MS/MS

Nehring A, Bury D, Kling H-W, Weiss T, Brüning T, Koch HM (2019) Determination of human urinary metabolites of the plasticizer di(2-ethylhexyl) adipate (DEHA) by online-SPE-HPLC-MS/MS. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1124:239–246. doi: 10.1016/j.jchromb.2019.06.019.

Abstract

Di(2-ethylhexyl) adipate (DEHA) is a plasticizer and phthalate substitute used in various consumer products. Relevant population exposures have to be assumed. In this study we describe the determination of three specific side chain-oxidized monoester metabolites of DEHA, mono-2-ethyl-5-hydroxyhexyl adipate (5OH-MEHA), mono-2-ethyl-5-oxohexyl adipate (5oxo-MEHA), and mono-5-carboxy-2-ethylpentyl adipate (5cx-MEPA) in human urine as potential biomarkers of DEHA exposure. After enzymatic hydrolysis, urine samples were analyzed by online turbulent flow chromatography for matrix depletion and analyte enrichment coupled to liquid chromatography-electrospray ionization-triple quadrupole-tandem mass spectrometry (online-SPE-LC-MS/MS). For quantification stable isotope dilution was applied with limits of quantification of 0.05 µg/L for 5cx-MEPA and 5OH-MEHA, and 0.1 µg/L for 5oxo-MEHA. Method accuracies (relative recoveries) were between 92-109%, and relative standard deviations <5%. We investigated the applicability of the method for internal DEHA exposure assessment in six volunteers who had consumed food wrapped in commercial PVC-cling film containing DEHA and in two small pilot populations without known DEHA exposure (44 pregnant Brazilian women and 32 German adults). In the cling film experiment, we could quantify all three metabolites in all post exposure urine samples, with 5cx-MEPA being most prominent (0.30 – 10.2 µg/L), followed by 5OH-MEHA (0.12 – 4.31 µg/L) and 5oxo-MEHA (0.12 – 2.84 µg/L). In the Brazilian and German samples we could detect DEHA exposure in 43 and 9% of all samples, again with 5cx-MEPA as the most prominent metabolite. Based on validation and pilot biomonitoring results, the method has proven appropriate for DEHA biomonitoring and will be applied in future metabolism and population studies.

4.1. Introduction

Bis(2-ethylhexyl) adipate (di(2-ethylhexyl) adipate, DEHA (synonym: Dioctyladipate (DOA)); CAS registry no. 103-23-1; EC no. 203-090-1) is used as a plasticizer in PVC products, surface coatings, adhesives and rubber. In several parts of the world, DEHA is permitted for use in food contact material [1–3] and has a wide range of applications in consumer and professional use, e.g. flooring and wall coverings, paints and lacquers, lubricants, washing and cleaning products or in the medical sector [4–7]. In 2012, DEHA production amounted to 18,000 t in the U.S. and 14,000 t in Western Europe [8].

DEHA can be a substitute for the plasticizer di(2-ethylhexyl) phthalate (DEHP) which has come under scientific and regulatory scrutiny because of its toxicity to reproduction and endocrine disrupting effects, resulting in use restrictions in many parts of the world (United States, 2008 [9] European Union (EU), 1999 [10]) and in a restriction of DEHP in the EU since 2015 [11]. Further broad restrictions on DEHP that now ultimately will also affect imported articles have been published recently [12]. DEHA is considered as a safer alternative to DEHP. DEHA is included in the EU ECHA Community Rolling Action Plan (CoRAP), a substance evaluation is expected to be started in 2020 [13]. Contrary to DEHP, for DEHA no anti-androgenic effects have been observed in rats [14,15] and DEHA showed no testicular toxicity in an induced rat liver damage model [16] or under conditions of renal dysfunction in rats [17]. In a repeated dose toxicity study where DEHA was applied for 28 days by gavage, Miyata et al. [12] reported increased kidney weights in males at the mid dose (200 mg/kg bodyweight (bw)/day) and liver weight increases at the top-dose level (1000 mg/kg bw/day) for males and females. In this study a no observed adverse effect level (NOAEL) of 40 mg/kg bw/day was identified. A developmental toxicity study with pre- and postnatal DEHA application by gavage identified a NOAEL of 200 mg/kg bw/d based on postnatal death. In this study, DEHA did not induce antiandrogenic effects similar to those of di(2-ethylhexyl) phthalate [15]. A NOAEL of 200 mg/kg bw/d for female fertility toxicity, based on histopathological changes in the ovary and disturbed ovulation in orally dosed rats, was reported by Wato et al. [18]. Based on a no observed effect level (NOEL) of 30 mg/kg bw for fetotoxicity in a teratogenicity study, the EU Scientific Committee on Food (SCF) derived a tolerable daily intake (TDI) of 0.3 mg/kg bw/d [19] and a specific migration limit (SML) of 18 mg/kg for food contact material [20]. Depending on several factors such as temperature and storage time and fat content of the food, the SML has been reported to be exceed in some cases [21–23]. Thus, due to its environmental occurrence, e.g. in rivers [24] or waste water [25], its reported presence in the indoor environment like in indoor air

or in dust [26,27], its direct use in consumer products, and in food contact material, population exposures to DEHA are very likely.

Takahashi et al. [28] investigated the elimination of ^{14}C-labeled DEHA in rats after oral administration. Up to 60% of the DEHA dose was excreted within 48 h post-dose via respiratory carbon dioxide. The remaining amount was excreted mainly in urine, predominantly as adipic acid. Loftus et al. [29] investigated DEHA metabolism in humans after oral exposure with DEHA that was deuterium labelled in the 2-ethylhexyl side chains, but only analyzed for, and detected 2-ethylhexanoic acid side chain derived ester cleavage products in urine. These products, however, are not unique to DEHA, but can be generated from any other xenobiotics having a 2-ethylhexyl ester moiety, such as DEHP or di-(2-ethylhexyl) terephthalate (DEHTP). Likewise, adipic acid is a metabolite of all adipates, and not unique for DEHA. In the quest for metabolites to be used as specific biomarkers of DEHA exposure Silva et al. [30] tentatively identified two specific 2-ethylhexyl sidechain-oxidized monoester metabolites *in vitro* with human liver microsomes: mono-2-ethylhydroxyhexyl adipate (OH-MEHA) and mono-2-ethyloxohexyl adipate (oxo-MEHA). They could also identify these metabolites in approximately 20% of urine samples from 144 adults from the general US population. These DEHA metabolites have in the meantime also been found in human urine by other research groups [31,32].

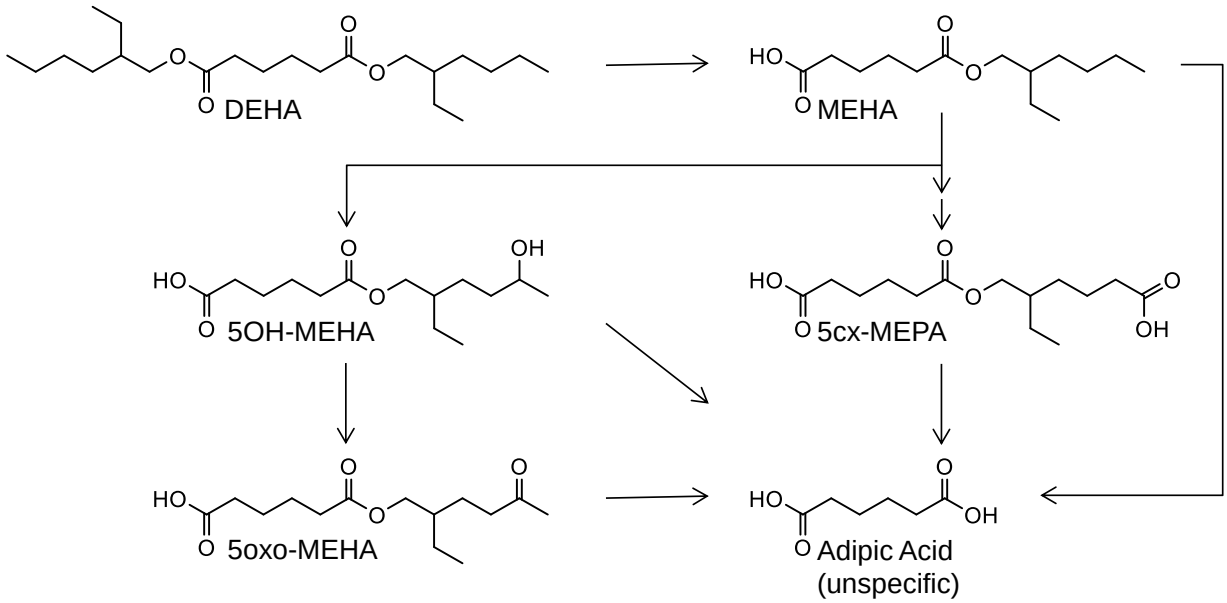

Fig. 1: *Postulated metabolism of DEHA in humans leading to the three specific, side chain oxidized metabolites 5OH-MEHA, 5oxo-MEHA and 5cx-MEPA and unspecific adipic acid. For simplification, phase two metabolites (e.g. glucuronic acid conjugates) are not shown*

Based on these tentatively identified specific DEHA metabolites and our experience with structurally similar analogs, such as DEHP, DEHTP, and EHS [33–35], we postulated the formation of the above two specific side chain oxidized metabolites of DEHA with oxidative modifications at position 5 of the hexyl chain: 1-mono-(2-ethyl-5-hydroxyhexyl) adipate (5OH-MEHA), and 1-mono-(2-ethyl-5-oxohexyl) adipate (5oxo-MEHA), including a newpotential metabolite, oxidized at the terminal position of the side chain: 1-mono-(2-ethyl-5-carboxylpentyl) adipate (5cx-MEPA). The postulated metabolism of DEHA leading to these three specific metabolites (including total breakdown to adipic acid) is depicted in **Fig. 1**. To allow rugged quantification, we decided to obtain not only (non-labeled) analytical standards but also authentic stable isotope-labeled internal standards for each of the three metabolites by custom synthesis. To prove the applicability of the method, we analyzed a small selection of urine samples from individuals with known DEHA exposure (food wrapped in DEHA-containing cling film) and from the general population (Brazil and Germany) without known exposures. In a next step, this method will be used in a quantitative study on human DEHA metabolism to derive urinary excretion factors for these DEHA metabolites, as previously exercised for several phthalates and their substitutes [33,34,36].

4.2.1. Materials and Method

4.2.1. Chemicals

1-mono-(2-ethyl-5-hydroxyhexyl) adipate (5OH-MEHA; >95%), 1-mono-(2-ethyl-5-oxohexyl) adipate (5oxo-MEHA; >97%), 1-mono-(2-ethyl-5-carboxylpentyl) adipate (5cx-MEPA; >97%), and their $^{13}C_6$-labeled analogs (same purity as corresponding unlabeled chemicals), with the labels in the adipic acid moiety, were custom synthesized by Dr. Vladimir Belov (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). The identities and purities of all synthesized metabolite standards (labeled and non-labeled) were confirmed by ESI-MS and ^{1}H NMR. Water and acetonitrile (LC-MS grade) were purchased from Carl Roth (Karlsruhe, Germany). Ammonium acetate (>98%) was purchased from Sigma Aldrich (Steinheim, Germany). *β*-glucuronidase (arylsulfatase free) from *E. coli* K12 was purchased from Roche Diagnostics (Mannheim, Germany). Acetic acid and formic acid (puriss p.a) were purchased from Honeywell Riedel-de Haën (Seelze, Germany).

4.2.2. High Resolution (HR)–MS mass spectra

HR-MS product ion spectra of all analytical standards, $^{13}C_6$- labeled and non-labeled, were recorded with Q-Orbitrap-MS (Q Exactive Focus, Thermo Scientific, Bremen, Germany) in ESI negative mode (ion spray voltage 2.5 kV, heater temperature 412 °C, capillary gas 256 °C) at maximum resolution setting (R = 70,000). The instrument gases (nitrogen) were set as follows: sheath gas 47.5 arbitrary units, auxiliary gas 11.25 arbitrary units. S-Lens RF Level was set to 50.

4.2.3. Preparation of standard stock solutions

For each standard (5OH-MEHA, 5oxo-MEHA, 5cx-MEPA; each, ^{13}C-labeled and non-labeled), a stock solution (1 g/L) was prepared by dissolving 10 mg, weighed exactly, in acetonitrile and filling up to10 mL. For the internal standard mix, 100-fold dilutions of each internal standard stock solution were prepared in acetonitrile and 150 µL of each dilution were mixed and filled up to 10 mL with water. All solutions were stored at -20 °C in glass flasks, capped with screw caps with silicone/teflon septa until further use.

4.2.4. Sample collection and preparation

Urine samples were stored in 250 mL polyethylene containers at -20 °C. For processing, samples were equilibrated to room temperature and mixed to homogenize sample material. An aliquot of 300 µL urine was transferred into a 1.5 mL screw neck vial (Machery Nagel, Düren, Germany). 100 µL ammonium acetate buffer (1 M, pH = 6.0 – 6.4), 20 µL internal standard mix and 6 µL *β*-glucuronidase (premixed 1:1 with ammonium acetate buffer) were added. The vial was capped with a silicone / PTFE screw cap (VWR, Darmstadt, Germany) and samples were mixed by inverting several times. Samples were incubated in a water bath for 3 h at 37 °C. After incubation, 30 µL of formic acid were added and samples were frozen overnight at -18 °C to precipitate cryophobic proteins. Samples were thawed at room temperature and centrifuged (10 min at 1900 g). The supernatant was transferred into a new 1.5 mL vial and used for analysis. Calibration solutions and quality control materials were treated the same way. Urinary creatinine concentrations were determined in each sample by L.u.P. GmbH Labor- und Praxisservice (Bochum, Germany) as contract analysis.

4.2.5. Calibration procedure and quality control

Seven calibration standards were prepared by dilution of non-labeled standard stock solutions in water. The concentrations of the calibration solutions ranged between 0.05 µg/L and 50 µg/L. Calibration functions were calculated by weighted (concentration^{-1}) linear regression of quotients of peak areas (non-labeled divided by $^{13}C_6$-labeled) vs. metabolite concentrations. Quality control material was prepared from urine samples of an oral dosing study with 10 mg DEHA (publication in preparation). Different individual samples (with natively present target biomarkers) were pooled, to prepare quality control material at three different concentration levels of DEHA metabolites (Q_{low}, Q_{med} and Q_{high}). Quality control material was frozen, thawed, and filtered consecutively three times to precipitate proteins and to ensure homogeneity.

The control material was aliquoted in 1.5 mL screw cap vials and stored at -20 °C until further use. The precision of the method was determined by analyzing Q_{low}, Q_{med}, and Q_{high} nine times within one series (intra-day precision) and on nine different days (inter-day precision).

For determination of method accuracy and ruggedness, nine urine samples with creatinine concentrations ranging between 0.3 and 2.3 g/L were spiked at three different concentration levels (2, 15 and 30 µg/L) and analyzed with and without spiking.

4.2.6. Chromatographic Conditions

An Agilent Technologies LC 1200 system (Waldbronn, Germany) was used, consisting of a G1329A autosampler, a G1311A quaternary pump coupled with a G4225A vacuum degasser, a G1312A binary pump coupled with a G1322A vacuum degasser, and a G1316A thermostated column compartment with 6-port switching valve. The functional principle of the two column assembly is described in more detail by Koch et al. (2003) [37]. The quaternary pump was used as loading pump (connected to the autosampler) and the binary pump for chromatographic separation (connected to the analytical LC column via the 6-port switching valve). The gradient delay volumes according to the manufacturer´s specifications were 600 - 900 µL for the binary pump flow path and 1122 - 1422 µL for the quaternary pump flow path (including 322 µL gradient delay volume of the autosampler). For online sample clean-up and analyte enrichment we applied a phenyl-modified silica gel turbulent flow chromatography column (TurboFlow® Phenyl 50 x 0.5 mm; Thermo Scientific™, Franklin, MA, USA). Chromatographic

separation was conducted on a superficially porous phenylalkyl-modified silica gel column (Accucore™ Phenyl-X 150 × 3 mm, particle size 2.6 µm; Thermo Scientific™, Franklin, MA, USA with SecurityGuard™ C18 4 x 3 mm, Phenomenex, Aschaffenburg, Germany). Water (solvent A) and acetonitrile (solvent B), each containing 0.05% acetic acid were used as eluents. The solvent gradients used for analyte enrichment and for chromatographic separation are shown in **table 1** and **table 2**. The flow rate on the binary pump was kept at 300 µL/min. The injection volume was 25 µL and after injection the needle was dipped into a wash vial containing acetonitrile/water 50:50 (v/v). The column compartment was kept at 22 ±1 °C. Analytes were extracted from the urinary matrix and trapped on the clean-up column for three minutes. Afterwards, the 6-port valve was switched to allow transfer of analytes onto the analytical column in backflush-mode. After 2 min (five minutes of total runtime), the switching valve was set back to begin analysis on the chromatographic column and simultaneously flush and re-equilibrate the enrichment column.

Table 1: *Solvent gradient for the loading pump (matrix depletion and analyte enrichment)*

Time [min]	Flow [µL/min]	Eluent A [%]	Eluent B [%]
0	1500	100	0
4	1500	100	0
5	1000	100	0
12	500	5	95
14	100	5	95
22	100	100	0
23	500	100	0
24	1000	100	0
25	1500	100	0
27	1500	100	0

Table 2: *Solvent gradient for the chromatographic separation (binary LC pump)*

Time [min]	Eluent A [%]	Eluent B [%]
0	70	30
2.5	70	30
4	60	40
19	45	55
20	5	95
22	5	95
23	70	30
27	70	30

4.2.7. Mass Spectrometric Conditions

Detection of DEHA metabolites was performed on an AB Sciex 5500 QTrap mass spectrometer (Darmstadt, Germany) with negative ESI ionization in scheduled multiple reaction monitoring (MRM) detection mode (ion spray voltage -4.5 kV, source heater temperature 550 °C). The instrument gases (nitrogen) were set as follows: curtain gas 25 psi, nebulizer gas 55 psi, and heater gas 50 psi. Collision gas pressure was set to ‘medium’ setting. The entrance potential was -10 V and collision cell exit potential was -11 V. The MRM detection window was set to 40 s and target scan time to 0.3 s. Further MS parameters (declustering potentials (DP) and collision energies (CE)) were optimized manually for each analyte **(table 3)**. Analyst 1.6.2 was used for instrument control and MultiQuant 3.0.2 for quantitative data analysis (both Sciex, Darmstadt, Germany). For each analyte two specific mass transitions were chosen. The transition with the highest signal-to-noise-ratio in matrix was used for quantification (quantifier), the next best for verification (qualifier).

Table 3: *Time programmed MRM parameters for each analyte*

	t_R [min]	*m/z* precursor ion	*m/z* product ion	DP [V]	CE [eV]
5OH-MEHA					
quantifier	9.12	273	83	-98	-23
qualifier			127		-18
$^{13}C_6$-5OH-MEHA					
quantifier	9.12	279	86	-90	-45
qualifier			133		-18
5oxo-MEHA					
quantifier	10.15	271	83	-112	-22
qualifier			127		-15
$^{13}C_6$-5oxo-MEHA					
quantifier	10.15	277	88	-114	-24
qualifier			133		-15
5cx-MEPA					
quantifier	9.70	287	145	-87	-22
qualifier			101		-34
$^{13}C_6$-5cx-MEPA	9.70	293			
quantifier			151	-83	-21
qualifier			88		-24

MRM: multiple reaction monitoring t_r: retention time, DP: declustering potential, CE: collision energy

4.2.8. Urine samples for pilot human biomonitoring

To simulate consumer relevant DEHA exposures, two volunteers consumed with their breakfast a single gouda cheese block each (1 cm³) wrapped in DEHA containing cling film (brand: Scel-O-frais® obtained from a consumer market) and stored overnight in the fridge (+8 °C). Four volunteers consumed a self-prepared cheese/ham sandwich each, wrapped in the same cling film under same storage conditions overnight. All six volunteers collected a spot urine sample 2-8 h after consumption (the second or third urine void after consuming the breakfast). In a second approach, we obtained urine samples from the general German and Brazilian population: 32 spot urine samples (convenience spot samples) from German volunteers (age 26 to 65 (median 44), 24 females and 8 males, urinary creatinine 0.121 to

1.782 g/L), not occupationally exposed to DEHA, were collected in July 2016. The study protocol was registered with the Ethics Commission of the Faculty of Medicine of the Ruhr-University Bochum, Germany (Registry Numbers: 3867-10 and 15-5422). 44 urine samples from pregnant Brazilian women were sampled in 2015. Urine samples were collected over all three trimesters of pregnancy and subsequently pooled for each woman. This study was approved by the IRB of the Health Sector of the Federal University of Paraná (Approval Number: 34820214.9.0000.0102).

4.2.9. Statistics

Descriptive parameters of the concentrations such as median values and percentiles were calculated with Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

4.3. Results and discussion

4.3.1. Chromatographic Separation

During method development, different columns for online sample clean-up and analyte enrichment were tested (a C18-modified polymer-coated silica gel phase, a C8-modified silica gel phase on restricted access material, a copolymeric phase with hydrophilic and hydrophobic characteristics, three turbulent flow chromatography (TFC) columns with a phenyl-modified silica gel phase, and two different polymeric phases; for column dimensions, manufacturers, etc. see Supplementary Material). The analytes were completely retained using pure aqueous mobile phase on all columns tested. To achieve an efficient refocusing on the analytical column during analyte transfer, elution of the analytes from the enrichment column at low mobile phase organic contents was desirable. The lowest organic content for fast and complete elution of all analytes was achieved using the phenyl TFC column. Using this column, swift analyte transfer was achieved at 30% ACN (refocusing was optimized in the final method by increasing to 40% ACN at the end of analyte transfer). Several analytical columns with different phase chemistries were tested as well (pentafluorophenyl-modified, biphenyl-modified, C18-modified, and phenyl-alkyl-modified silica gel phases, and a graphitic carbon phase; for column dimensions, manufacturers, etc. see Supplementary Material). For optimal refocusing during analyte transfer, we sought for strong retention of the analytes on the analytical column, with the analyte eluting first (5OH-MEHA in all cases) being the limiting factor. On the C18 column 5OH-MEHA was eluted at the highest organic content

(45% ACN under the conditions of the preliminary experiment, i.e., 10% ACN/min; on the other columns 5OH-MEHA was eluted at around 40% ACN; the graphitic carbon column resulted in broad peaks (up to 1 min)) and was thus initially chosen. However, two chromatographic interferences resulted in overestimations of 5oxo-MEHA and 5cx-MEPA in some urine samples (as seen by quantifier/qualifier ratios differing from the calibration standards) which could not be separated using a low gradient slope of 1%/min. On the phenyl-alkyl-modified phase, however, these interferences were separated (see for example **fig. 3c** - small peak eluting in front of 5cx-MEPA) and quantifier/qualifier ratios were plausible for all urine samples analyzed.

4.3.2. Tandem mass spectrometry

Product ion spectra of 5OH-MEHA, 5oxo-MEHA, and 5cx-MEPA with postulated fragment structures are shown in **fig. 2** (for product ion spectra of $^{13}C_6$- labeled internal standards see supplementary material **fig. S1**). All postulated fragments described below were verified by their accurate masses using Q-Orbitrap-MS (below ±5 ppm), data not shown. For all three metabolites a characteristic fragment at *m/z* 145 was observed, which can be explained as the adipic acid anion. As described by Grossert et al. [38] for free adipic acid, *m/z* 145 can either eliminate water to form *m/z* 127 (in case of 5cx-MEPA, *m/z* 127 might also be formed by initial elimination of water from the adipic acid moiety, resulting in an alkyne oxide, followed by ester cleavage) or it can eliminate CO_2, resulting in *m/z* 101. The fragment at *m/z* 127 can eliminate either CO_2 to yield *m/z* 83 or formic acid to yield *m/z* 81. $^{13}C_6$- labeled analytes also form the same fragments, only found at accordingly higher masses (*m/z* 86, 88, 133, 151; see supplementary material **fig S1**). As already known from other structurally similar analogs, like DEHP [39], DINCH [40] or DEHTP [41], specific alkyl sidechain fragments were only observed in case of the carboxy metabolite 5cx-MEPA. For 5cx-MEPA and its $^{13}C_6$-labeled analog the side chain alcohol (5-(hydroxymethyl)heptanoic acid) anion was observed at *m/z* 159. Furthermore, elimination of formic acid from the 2-ethylcarboxypentyl moiety of $[M-H]^-$, followed by elimination of adipic acid anhydride yields *m/z* 113. The fragment at *m/z* 141 could be explained by elimination of adipic acid from $[M-H]^-$. Alternatively, it could be formed by initial elimination of water at the 2-ethylcarboxypentyl moiety, resulting in an alkyne oxide, followed by ester cleavage.

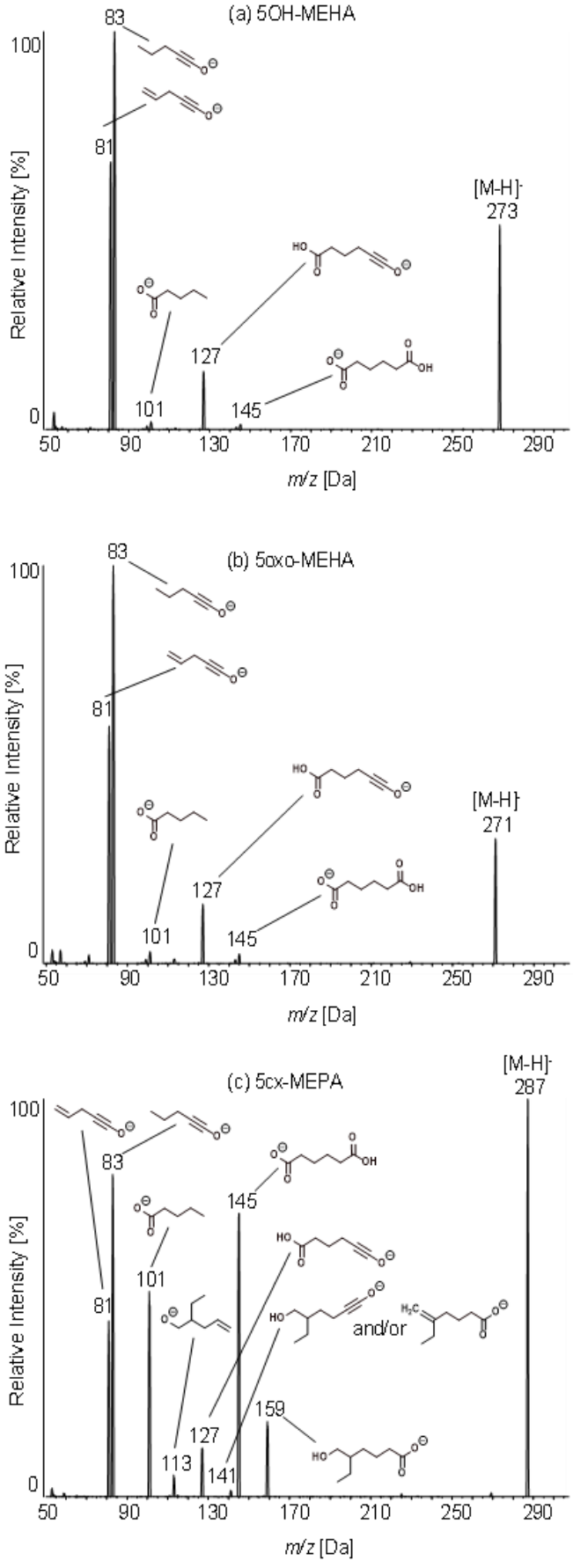

Fig.2: *Product ion spectra of 5OH-MEHA (a), 5oxo-MEHA (b), and 5cx-MEPA (c), recorded via direct infusion of each standard solution (1 mg/L dissolved in acetonitrile/water 50:50 (v/v), containing 0.05% acetic acid) at 7 µL/min*

4.3.3. Method performance

To prevent cleavage of the ester bond of specific DEHA monoester metabolites, which would result in unspecific hydrolysis products, we used a highly purified *β*-glucuronidase from *E. coli* K12. This particular enzyme preparation shows no measurable lipase/aryl sulfatase activity and is highly specific for *β*-glucuronides [37,42]. To find conditions for quantitative and robust deconjugation of all glucuronidated DEHA metabolites, Q_{high} was analyzed after incubation with *β*-glucuronidase for different time periods (0, 1, 2, 3, 4, 5 h). Also, as negative control, Q_{high} was analyzed without addition of enzyme. Compared to the negative control, increased metabolite concentrations were found in all enzyme-treated samples. Maximum metabolite concentrations were found at 1 h incubation time with longer incubation showing no further effect. An incubation time of 3 h was chosen for increased ruggedness. Calibration curves were linear throughout the complete calibration range (0.05 µg/L and 50 µg/L) for all metabolites ($r \leq 0.998$). Limits of quantification (LOQ) were estimated, based on a signal-to-noise ratio of 10 in matrix (for further details see Bury et al. [43]): 0.05 µg/L for 5cx-MEPA and 5OH-MEHA, and 0.1 µg/L for 5oxo-MEHA. Limits of detection (LOD), analogously derived based on a signal-to-noise ratio of 3, were: 0.02 µg/L for 5cx-MEPA and 5OH-MEHA, and 0.03 µg/L for 5oxo-MEHA. The method's imprecison was ≤5%, both intra- and inter-day (**table 4**). For evaluation of the method accuracy, relative recoveries were determined after subtraction of background levels (non-spiked sample). Relative recoveries ranged from 92% to 109% for all samples, metabolites and spiking levels (**table 5**). No relation between creatinine content and relative recovery was observed. We detected no interfering contamination for any of the target analytes in water or procedural blank samples. Furthermore, we detected no carry-over after the highest calibration standards into following blank samples.

Exemplary chromatograms of a procedural water blank sample (a), a calibration standard in water (b) and a processed native urine sample (c) are shown in **fig. 3.**

Table 4: *Precision data of the method. All quality control samples (Q_{low}, Q_{med}, Q_{high}) were prepared from urine samples containing native DEHA metabolites*

	5OH-MEHA			5oxo-MEHA			5cx-MEPA		
	Q_{low}	Q_{med}	Q_{high}	Q_{low}	Q_{med}	Q_{high}	Q_{low}	Q_{med}	Q_{high}
Intraday (n=9)									
Mean measured conc. [µg/l]	0.55	3.00	16.4	0.42	1.86	15.6	1.60	9.70	25.2
(range)	(0.54-0.57)	(2.91-3.08)	(16.1-16.9)	(0.41-0.43)	(1.81-1.90)	(15.3-15.9)	(1.58-1.65)	(9.54-9.88)	(24.8-25.4)
RSD [%]	1.7	1.8	1.7	2.1	1.6	1.3	1.6	1.2	0.7
Interday (n=9)									
Mean measured conc. [µg/L]	0.53	3.02	16.2	0.40	1.85	16.0	1.60	9.60	25.0
(range)	(0.51-0.55)	(2.76-3.13)	(15.9-16.6)	(0.37-0.42)	(1.77-1.92)	(15.6-16.7)	(1.53-1.64)	(8.48-10.09)	(24.2-25.6)
RSD [%]	1.9	3.7	1.3	4.1	2.8	2.6	1.9	4.9	1.67

RSD: relative standard deviation

Table 5: *Accuracy (relative recovery) calculated from analysis of nine different urine samples with different creatinine concentrations (0.34 to 2.3 g/L creatinine) and spiked with approximately 2 µg/L, 15 µg/L and 30 µg/L*

	5OH-MEHA			5oxo-MEHA			5cx-MEPA		
Native concentrations [µg/L]	<LOQ			<LOQ			<LOQ-0.07		
Spiked concentrations [µg/L]	2.14	17.8	35.6	1.79	14.9	29.9	1.89	15.7	31.5
Measured concentrations[a] [µg/L]	2.10	18.8	36.9	2.45	15.0	29.6	1.92	16.3	31.1
Mean (range)	(2.02-2.19)	(18.5-19.2)	(34.9-38.4)	(2.01-4.12)	(14.7-15.4)	(28.2-30.5)	(1.76-2.03)	(15.9-17.3)	(29.0-33.4)
Accuracy [%]	98	106	104	97	100	99	101	104	99
Mean (range)	(95-103)	(104-108)	(98-108)	(92-104)	(98-103)	(94-102)	(93-107)	(101-109)	(92-106)

[a] background levels substracted; LOQ: limit of quantification (5cx-MEPA and 5OH-MEHA: 0.05 µg/L, 5oxo-MEHA: 0.1 µg/L)

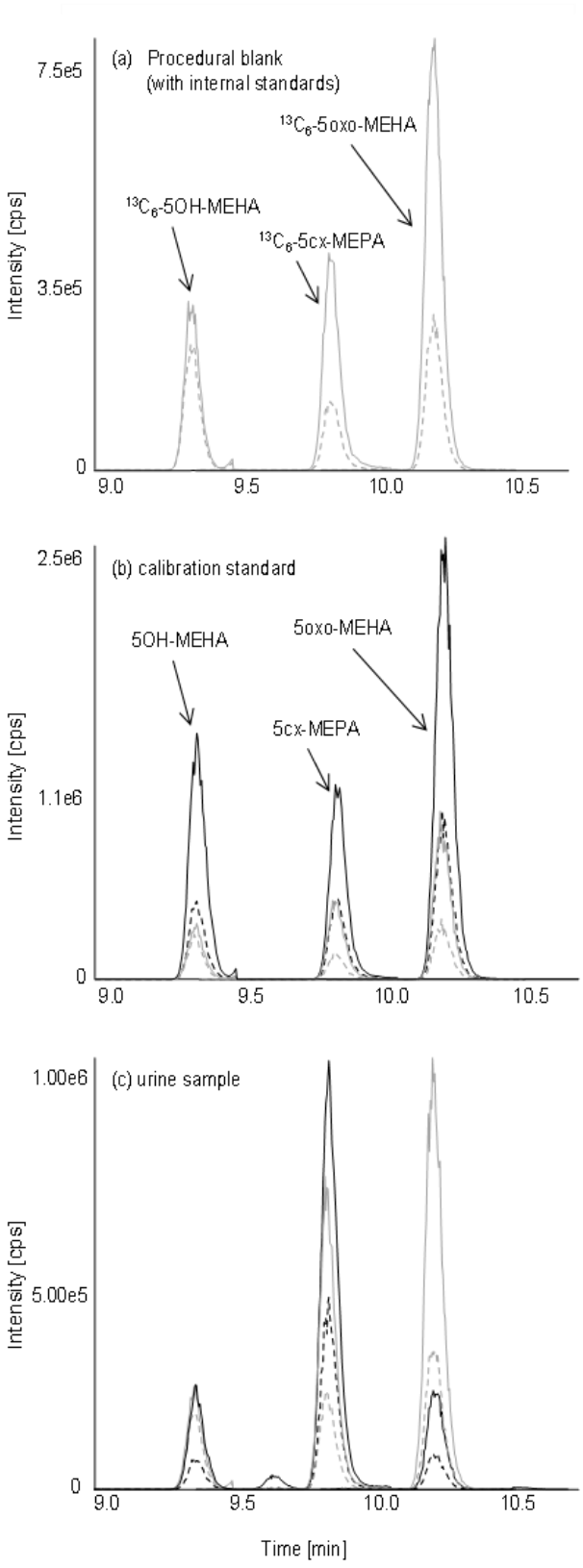

Fig. 3: *Chromatograms of a processed procedural blank solution including internal standards of each metabolite (a), a processed calibration standard solution in water containing each analyte at ~15 µg/L (b), and a urine sample collected 6 h after consumption of PVC-cling film wrapped food with the following metabolite concentrations: 5OH-MEHA: 3.68 µg/L, 5cx-MEPA: 10.2 µg/L, 5oxo-MEHA: 2.8 µg/L (c). Non-labeled metabolites in black and internal standards in gray; quantifier transitions as continuous lines and qualifier transitions in dotted lines*

4.3.4. Biomonitoring Results

In the urine samples of all six volunteers who had eaten food (cheese and sandwiches) wrapped in PVC cling film overnight, all three DEHA metabolites were detected above the LOQ (see exemplary chromatogram **Fig. 3 (c)**). 5cx-MEPA was present in highest concentrations (median: 1.05 µg/L; range: 0.30-10.2 µg/L) followed by 5OH-MEHA (0.56 µg/L; 0.12-4.31 µg/L) and 5oxo-MEHA (0.36 µg/L; 0.12-2.84 µg/L) (see **table 6**). We observed highest metabolite concentrations in the two individuals who had consumed the cling film wrapped cheese. Metabolite concentrations in the four volunteers, who had consumed the cling film wrapped sandwiches, were generally lower by a factor of ~5-40 (see supplementary material **table S1**).

In the two pilot populations without known DEHA exposure, we could quantify one or more DEHA metabolites in 43.2% of the pooled samples from pregnant Brazilian women (5cx-MEPA: 43.2%, 5OH-MEHA: 9.1%, 5oxo-MEHA: 4.5%) but only in 9.4% of the samples from the German adult volunteers (5cx-MEPA = 9.4%), see **table 6**. Again, we detected 5cx-MEPA at highest concentrations, followed by the other metabolites at lower concentrations. DEHA exposures for the German population seemed somewhat lower and less prevalent than for Brazil. In both pilot populations, the maximum urinary level of 5cx-MEPA (0.24 µg/L) was below the lowest 5cx-MEPA level observed in the cling-film scenario from above (0.30 µg/L).

Table 6: *Results of the Pilot Biomonitoring Study*

	volunteer study ("cling film exposure")[a]			**pregnant Brazilian women**[b]			**German adults**[c]		
	5cx-MEPA	5OH-MEHA	5oxo-MEHA	5cx-MEPA	5OH-MEHA	5oxo-MEHA	5cx-MEPA	5OH-MEHA	5oxo-MEHA
>LOQ [%]	100	100	100	43.2	9.1	4.5	9.4	0	0
Median [µg/L][d]	1.05	0.56	0.36	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
P 95 [µg/L][d]	9.80	4.15	2.70	0.20	0.06	<LOQ	0.06	<LOQ	<LOQ
Range [µg/L]	0.30 - 10.2	0.12 - 4.31	0.12 - 2.84	<LOQ - 0.24	<LOQ - 0.07	<LOQ - 0.13	<LOQ - 0.24	<LOQ	<LOQ

[a]n=6, [b]n=44, , [c]n= 32 [d]Median and 95th percentile (P 95) concentrations not reported, if 50% and 95%, respectively, of the samples were below limit of quantification (LOQ)

In 2013, Silva et al. [30] provided first but tentative (no authentic analytical standards) biomonitoring data on specific DEHA metabolites in human urine samples (n = 144) from the USA. They also detected OH-MEHA and oxo-MEHA, but in less than 20% of the samples. Urinary concentrations reported by Silva et al. [30] ranged between <LOD (0.2 µg/L)-23.9 µg/L for OH-MEHA (sum of isomers), and <LOD (0.2 µg/L)-10.4 µg/L for oxo-MEHA (sum of isomers). These concentrations are slightly higher than the concentrations determined in our cling film investigation, and considerably higher than maximum concentrations from our two pilot populations, but in line with our results of confirming background exposures of the general population to DEHA. Unfortunately, Silva et al. [30] did not include 5cx-MEPA in their investigations, which has been identified by us to be the most prominent of the three side chain oxidized metabolites.

Overall, considering the LOQs of our newly developed method, our pilot biomonitoring investigations show that 5cx-MEPA is potentially the most sensitive exposure biomarker to specifically quantify DEHA exposure. Our new method is capable of detecting DEHA exposures both after consumption of presumably DEHA contaminated foodstuff, and in broader study populations without known or presumed exposure to DEHA.

4.4. Conclusions

With the analytical method described in this study we fulfilled our foremost aim to specifically detect and quantify DEHA exposure down to levels observable in urine samples from the general population. For the first time, 5cx-MEPA was analyzed as specific metabolite of DEHA in human urine. We could show that this metabolite is excreted at considerably higher concentrations compared to 5oxo-MEHA and 5OH-MEHA, thus being a potentially more sensitive biomarker of DEHA exposure. Furthermore, authentic stable isotope-labeled internal standards of oxidized DEHA metabolites have been applied for the first time. Other urinary metabolites of DEHA (not included in this method) such as adipic acid or metabolites derived from the sidechain (e.g. 2-ethylhexanoic acid) may be excreted at higher concentrations, but are not specific to DEHA as they can be generated from other adipic acid or ethylhexanol-containing molecules. The LOQs were sufficiently low to detect DEHA exposures in all urine samples collected after simulating consumer related exposures (consumption of cling film wrapped food) and in a noticeable number of urine samples from the general population with no known DEHA exposure from Germany and Brazil. Thus, this methodology is suitable to investigate possible

differences in DEHA exposure depending on the region, depending on the year of collection (time-trends) or depending on specific sub-populations (such as children) as previously exercised for other plasticizers [44–48]. In a next step, this method will be used to quantitatively investigate human metabolism and renal excretion of DEHA after defined dosage (publication in preparation) in order to derive urinary metabolite excretion fractions (F_{ue}s). These F_{ue}s can then be used to reliably back-calculate DEHA intake levels from urinary metabolite levels and thus support an informed exposure and risk assessment for DEHA.

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4.6. Compliance with ethical standards

Declarations of interest: None.

4.7. References

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Supplementary Material

S4.1. Further information about Chromatographic Separation

The following columns for online sample clean-up and analyte enrichment were tested during method development:

Capcell PAK® C18 MG-II (10 x 4 mm, particle size 5 µm; Phenomenex®, Aschaffenburg, Germany)

LiChrospher® RP-8 ADS RAM (25 x 4 mm, particle size 25 µm; Merck, Darmstadt, Germany)

Oasis® HLB (20 x 2.1 mm, particle size 5 µm; Waters, Eschborn, Germany)

TurboFlow® Phenyl (50 x 0.5 mm; Thermo ScientificTM, Franklin, MA, USA) *

TurboFlow® Cyclone-P (50 x 0.5 mm; Thermo ScientificTM, Franklin, MA, USA)

TurboFlow® Cyclone (50 x 0.5 mm; Thermo ScientificTM, Franklin, MA, USA)

The following analytical columns were tested during method development:

Luna® PFP (150 x 2 mm, particle size 3 µm; Phenomenex®, Aschaffenburg, Germany)

Kinetex® Biphenyl (150 x 3 mm, particle size 2.6 µm; Phenomenex®, Aschaffenburg, Germany)

Kinetex® C18 (150 x 3 mm, particle size 2.6 µm; Phenomenex®, Aschaffenburg, Germany)

AccucoreTM Phenyl-X (150 x 3 mm, particle size 2.6 µm; Thermo ScientificTM, Franklin, MA, USA) *

HypercarbTM (100 x 2.1 mm, particle size 5 µm; Thermo ScientificTM, Franklin, MA, USA)

*: used in the final method

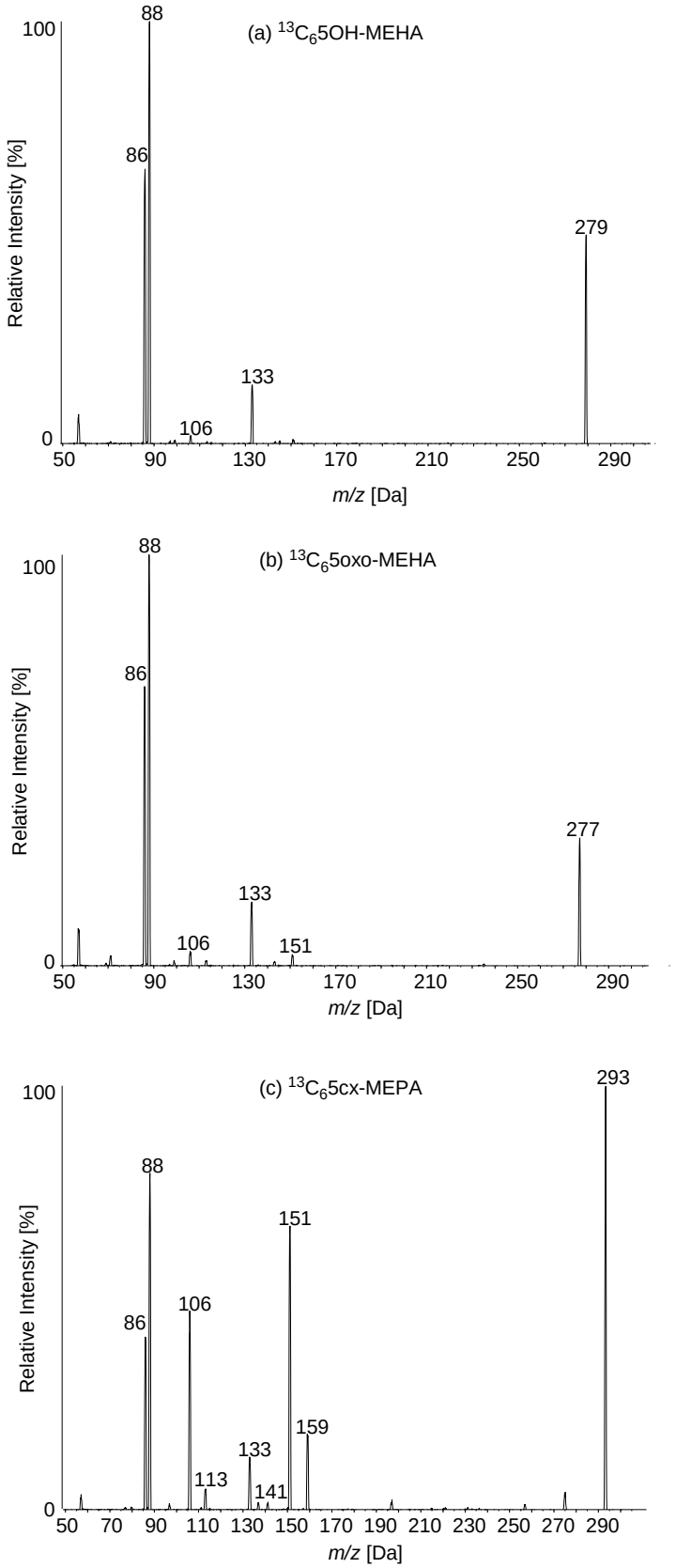

***Fig. S1**: Product ion spectra of $^{13}C_6$ labeled DEHA metabolites, recorded via direct infusion of each standard solution (1 mg/L dissolved in acetonitrile/water 50:50 (v/v), containing 0.05% acetic acid) at 7 µL/min*

***Table S1**: Concentration of DEHA metabolites in urine of volunteers, who had eaten food wrapped in DEHA containing cling film overnight. Volunteer1 and 2 ate a cheese block, the remaining volunteers consumed self-prepared sandwiches*

	German adults ("cling film exposure")		
	5cx-MEPA [µg/L]	5OH-MEHA [µg/L]	5oxo-MEHA [µg/L]
Volunteer 1	10.15	4.31	2.84
Volunteer 2	8.73	3.68	2.27
Volunteer 3	0.51	0.33	0.34
Volunteer 4	0.3	0.12	0.13
Volunteer 5	1.59	0.78	0.37
Volunteer 6	0.36	0.19	0.12

Kapitel 5

Metabolism and urinary excretion kinetics of di(2-ethylhexyl) adipate (DEHA) in four human volunteers after a single oral dose

Nehring A, Bury D, Ringbeck B, Kling H-W, Otter R, Weiss T, Brüning T, Koch HM (2020) Metabolism and urinary excretion kinetics of di(2-ethylhexyl) adipate (DEHA) in four human volunteers after a single oral dose. Toxicol. Lett. 321:95–102. doi: 10.1016/j.toxlet.2019.12.006

Abstract

Di(2-ethylhexyl) adipate (DEHA) is used as a substitute for the reprotoxic phthalate plasticizer di(2-ethylhexyl) phthalate (DEHP). This study reports the first quantitative data on human *in vivo* DEHA metabolism and urinary metabolite excretion with the aim of providing tools for DEHA exposure and risk assessments. After DEHA was administered to four healthy volunteers (107-164 µg/kg body weight (bw)), urine samples were continuously and completely collected for 48 h and analyzed for the specific oxidized monoester metabolites mono-2-ethyl-5-hydroxyhexyl adipate (5OH-MEHA), mono-2-ethyl-5-oxohexyl adipate (5oxo-MEHA), and mono-5-carboxy-2-ethylpentyl adipate (5cx-MEPA), as well as for the non-specific hydrolysis product adipic acid (AA) using stable isotope dilution analysis. AA was confirmed as a major (urinary excretion fraction (F_{UE}): 10-40%), yet non-specific DEHA metabolite. 5cx-MEPA was the major specific DEHA metabolite with an F_{UE} of 0.20% (range: 0.17-0.24%). F_{UE}s for 5OH-MEHA and 5oxo-MEHA were 0.07% (0.03-0.10%) and 0.05% (0.01-0.06%), respectively. The three specific metabolites were excreted with two concentration maxima (t_{max1} = 1.5-2.3 h, t_{max2} = 3.8-6.4 h). Elimination half-lives ($t_{1/2}$, calculated after the second t_{max}) for 5cx-MEPAwere calculated between 2.1 to 3.8 h. The majority (98-100%) of metabolites was excreted within 24 hours. The F_{UE} of 5cx-MEPA was applied to demonstrate its applicability for calculating daily intakes based on urinary metabolite levels from three pilot populations. Daily intakes were generally far below the tolerable daily intake (TDI) for DEHA (300 µg/kg bw/day). The highest daily intake (114 µg/kg bw/day) was calculated in individuals after consuming food that had been wrapped in DEHA containing cling film.

5.1. Introduction

Di(2-ethylhexyl) adipate (DEHA; synonyms: bis(2-ethylhexyl) adipate and dioctyladipate (DOA); CAS registry no. 103-23-1; EC no. 203-090-1) is a plasticizer which improves low-temperature flexibility of polymeric products (Stuer-Lauridsen et al. 2001; BASF 2012; Malveda et al. 2015). It is used in different commercial and industrial applications, such as in flooring and wall coverings, paints and lacquers, PVC toys, and medical devices (BASF 2012; Stuer-Lauridsen et al. 2001; Norwegian Environmental Agency et al.; Biedermann-Brem et al. 2008; Gimeno et al. 2014; Malarvannan et al. 2019). In several parts of the world (e.g. United States (U.S.), European Union (EU)) DEHA is also permitted for use in food contact materials (Malveda et al. 2015; U.S. Food and Drug Administration (FDA) 2019; European Commission 2019). DEHA consumption in 2014 amounted to 24,000 t in the U.S. and 14,000 t in Western Europe (Malveda et al. 2015).

DEHA is applied to products as an alternative to the ortho-phthalate plasticizer di(2-ethylhexyl) phthalate (DEHP), which is subject to bans and use restrictions in many countries due to its reproductive toxicity and endocrine disrupting effects, e.g. in the U.S. since 2008 (Consumer Product Safety Commission 2008) and the EU since 1999 (European Commission 1999; European Parliament and the Council of the European Union 2005, 2006, 2009; European Commission 2011, 2018). Overall, DEHA is considered a safer alternative to DEHP. In some studies, DEHA was found to affect the female reproductive system at high doses (Dalgaard et al. 2003; Miyata et al. 2006; Wato et al. 2009). After single ip doses of DEHA spermatogenesis was affected in male mice (Singh et al. 1975). However, neither the anti-androgenic effects (Dalgaard et al. 2003; Miyata et al. 2006) nor the testicular toxicity attributed to DEHP has been observed in rats (Kang et al. 2006; Nabae et al. 2006). A substance evaluation addressing suspected CMR properties, consumer use, high aggregated tonnage and wide dispersive use is expected to be started in 2020 (European Chemicals Agency (ECHA)) within the Community Rolling Action Plan (CoRAP) of the EU. For now, based on a no observed effect level (NOEL) of 30 mg/kg bodyweight (bw)/day in a teratogenicity study, the EU Scientific Committee on Food (SCF) derived a tolerable daily intake (TDI) of 0.3 mg/kg bw/day for DEHA and a specific migration limit (SML) of 18 mg/kg food (European Commission 2000). Several studies identified food as a relevant exposure source for DEHA, most likely due to the use in food contact materials (Castle et al. 1987; Tsumura et al. 2001; Fromme et al. 2007; Fromme et al. 2013; Cao et al. 2015) and exceedance of the SML for DEHA depending on storage conditions (time, temperature) and fat content of the food has

been shown (Daun and Gilbert 1977; Castle et al. 1987; Till et al. 1987; Højslev Petersen and Tubæk Naamansen 1998). Given its use in consumer products,food contact materials and medical devices (Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIHR) 2016), and considering its presence in the aquatic environment (Horn et al. 2004; Barnabé et al. 2008) and in indoor environments (e.g. indoor air and dust) (Fromme et al. 2016; Subedi et al. 2017; Christia et al. 2019; Giovanoulis et al. 2019), exposures of the general population to DEHA are highly likely.

Human biomonitoring (HBM) is a well-established tool for conducting integral (covering all exposure sources and uptake routes) exposure assessments, both on an individual and population level (Needham et al. 2007; Schindler et al. 2014; Haines et al. 2017; Kolossa-Gehring et al. 2017; Schwedler et al. 2017). For several phthalate-type plasticizers and non-phthalate plasticizer substitutes, exposure biomarkers are established and have been applied for exposure assessments in large-scale population studies (Zota et al. 2014; Koch et al. 2017; Silva et al. 2017; Kasper-Sonnenberg et al. 2019; Lessmann et al. 2019). However, for DEHA exposure biomarkers applicable to the general population have not been fully established, yet. Silva et al. (2013) tentatively identified three DEHA metabolites *in vitro*: the simple monoester mono-2-ethylhexyl adipate (MEHA) and its downstream metabolites mono-2-ethylhydroxyhexyl adipate (OH-MEHA) and mono-2-ethyloxohexyl adipate (oxo-MEHA) and detected these metabolites in urine samples from U.S. individuals, which was later confirmed by other groups in European populations (Bastiaensen et al. 2019; Been et al. 2019; Pinguet et al. 2019). Recently, we presented an analytical method for the robust determination of the specific isomers of these DEHA metabolites 5OH-MEHA (mono-2-ethyl-5-hydroxyhexyl adipate) and 5oxo-MEHA (mono-2-ethyl-5-oxohexyl adipate), as well as the not previously described carboxy metabolite 5cx-MEPA (mono-5-carboxy-2-ethylpentyl adipate) in human urine using authentic standards and stable isotope labeled internal standards (Nehring et al. 2019). From these three potential DEHA metabolites, 5cx-MEPA was found most frequently in urine samples from a Brazilian and a German pilot population (43% and 9% above the limit of quantification (LOQ), respectively) and at considerably higher concentrations than the respective OH- and oxo-metabolites. The same metabolite ratios were observed in a staged DEHA exposure scenario, where six volunteers consumed food wrapped in DEHA containing PVC-cling film (Nehring et al. 2019).

In the current study we quantitatively investigated the formation and urinary excretion of 5OH-MEHA, 5oxo-MEHA and 5cx-MEPA in humans after a single oral dose, with the aim of providing toxicokinetic data for future DEHA exposure and risk assessments in population based HBM studies. These data include urinary excretion fractions (F_{UE}) which can be used to derive external doses (daily intakes (DI)) from urinary metabolite concentrations (Angerer et al. 2011; Hays and Aylward 2012; Apel et al. 2017) which can be related to toxicological threshold values, such as the tolerable daily intake (TDI) for DEHA (for examples see Hartmann et al. 2015; Koch et al. 2017; Correia-Sá et al. 2017; Ulrich et al. 2018; Lessmann et al. 2019).

5.2. Materials and methods

5.2.1. Chemicals

Di(2-ethylhexyl) adipate (≥99.5%) used in the oral dosing study was provided by BASF SE (Ludwigshafen, Germany). Adipic acid (certified reference material) was purchased from Sigma Aldrich (Steinheim, Germany), and $^{13}C_6$-labeled adipic acid (99%) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Ethanol (≥99.8%) was purchased from Honeywell Riedel-de Haën (Seelze, Germany). Ammonium formate (LC-MS grade) was purchased from Honeywell Fluka™ (Bucharest, Romania). Pure β-glucuronidase from *E. coli* K12 without arylsulfatase/esterase activity (Roche Diagnostics, Mannheim, Germany) was used for deconjugation of phase II metabolites. For further information on chemicals and reagents see Nehring et al. (2019).

5.2.2. Study design

Four healthy German volunteers (2 females, 2 males; aged between 24 and 34 years; bodyweight between 59 and 91 kg), without known occupational exposure to DEHA, each received a single oral dose of approximately 10 mg (weighed precisely) of DEHA. The volunteers received the dose dissolved in 1 mL ethanol and diluted with water, administered in a chocolate coated waffle cup, followed by a small breakfast. The resulting individual doses ranged from 107 to 164 µg/kg bw and were well below the TDI for DEHA of 0.3 mg/kg bw/day derived by the SCF. Furthermore, one of the four volunteers

additionally received an equimolar dose of MEHA (6.73 mg; corresponding to 72.3 µg/kg bw) in the same manner several weeks after the DEHA dose.

The first urine sample (full void) was provided immediately before dose (t= 0). After dose, urine samples were collected subsequently and completely over 48 hours. Sampling times were noted by the volunteers. Urine samples were collected in 250 mL polyethylene containers and sample volumes were determined by the mass difference between empty and filled sample containers. Samples were stored frozen at -20 °C until further use. Urinary creatinine concentrations were determined by L.u.P GmbH Labor- und Praxisservice (Bochum, Germany).

Approval for the study protocol was obtained from the Ethics Commission of the Faculty of Medicine of the Ruhr-University Bochum, Germany (IRB Reg. No. 15-5422) and written informed consent was obtained from each volunteer.

5.2.3. Quantification of the monoester metabolites 5cx-MEPA, 5OH-MEHA, and 5oxo-MEHA

Determination of secondary oxidized DEHA metabolites (5cx-MEPA, 5OH-MEHA, and 5oxo-MEHA) in urine performed in this study has been previously described by our group (Nehring et al. 2019). In brief, urine samples underwent enzymatic hydrolysis with pure β-glucuronidase from *E. coli* K12 (without arylsulfatase/esterase activity) followed by liquid chromatography-electrospray ionization-triple quadrupole-tandem mass spectrometry with online turbulent flow chromatography for matrix depletion and analyte enrichment (online-SPE-LC-MS/MS). Limits of quantification (LOQ) were 0.05 µg/L for 5cx-MEPA and 5OH-MEHA, and 0.1 µg/L for 5oxo-MEHA.

5.2.4. Quantification of adipic acid

Urine samples were diluted 100-fold with water and 300 µL were mixed with 100 µL ammonium acetate buffer (1 M, pH = 6.0 – 6.4), 30 µL internal standard solution ($^{13}C_6$-adipic acid in water), and 6 µL β-glucuronidase (premixed 1:1 with ammonium acetate buffer). After enzymatic deconjugation (3 h, 37 °C), 30 µL formic acid were added. 5 µL of the processed sample were analyzed with LC-MS/MS. A reversed phase column with superficially porous particles (Kinetex C18 150 x 3.0 mm, particle size 2.6 µm, with corresponding SecurityGuard; Phenomenex, Aschaffenburg, Germany) was used for

chromatographic separation and detection was performed using a 5500 triple quadrupole mass spectrometer with electrospray ionization (ESI) in negative ion mode (AB Sciex, Darmstadt, Germany). The LOQ was 1 µg/L in the diluted samples, corresponding to 100 µg/L in the non-diluted urine. For further information see the Supplementary Material.

5.2.5. Metabolite screening for additional urinary metabolites

Urine samples after the single DEHA dose from one volunteer up to 8 h after dose were analyzed for putative DEHA metabolites using three different suspect screening approaches: liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), an approach with online solid-phase extraction connected with LC-MS/MS (online-SPE-LC-MS/MS) adapted from Bury et al. (2019a; 2019b) using two turbulent flow chromatography (TFC) columns (one silica-based phenyl phase and one polymer-based phase) in series, and an online-SPE-LC-MS/MS approach using a mixed mode (reversed phase and anion exchange) TFC column. For the postulated metabolites two mass transitions were analyzed. For further information see the Supplementary Material.

5.2.6. Statistical analysis

All the data analysis in this study was conducted on Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). After the single DEHA dose, urinary metabolite concentrations (c) past the second metabolite concentration maximum c_{max2} can be described by the equation $c(t) = c_{max2} * e^{-kt}$, with k being the kinetic constant of this exponential decline. For the calculation of the elimination half-life $t_{1/2}$ for 5cx-MEPA, the kinetic constant k was obtained by exponential regression of metabolite concentrations c(t) past c_{max2} vs. t. The elimination half-life $t_{1/2}$ was calculated using the equation $t_{1/2} = \ln(2)/k$ (Byers and Sarver 2009).

Urinary excretion fractions (F_{UE}) (i.e., the percentage of the applied dose which is excreted as the respective metabolite) were calculated as per the following equation (with $\sum m_i$ the sum of masses of the respective metabolite in all urine samples, M(DEHA) and M(Metabolite) the molar masses of DEHA and the respective metabolite, and D the applied DEHA dose):

$$F_{UE} = \frac{\sum m_i * \left(\frac{M(DEHA)}{M(Metabolite)}\right)}{D} * 100\%$$

To account for high background AA concentrations, F_{UE}s for this metabolite were calculated with background correction. For further information see the Supplementary Material. Daily intakes (DI) calculations were performed according to Kohn et al. (2000) and Koch et al. (2003), using creatinine excretion rates reported by Harper et al. (1977).

5.3. Results and discussion

5.3.1. DEHA metabolites under investigation

Based on previous findings (Silva et al. 2013) and experience with structurally similar xenobiotics, such as DEHP, di(2-ethylhexyl) terephthalate (DEHTP), and 2-ethylhexyl salicylate (EHS) (Koch et al. 2005; Lessmann et al. 2016; Bury et al. 2019a), the putative metabolites MEHA, 5OH-MEHA, 5oxo-MEHA, and 5cx-MEPA have been postulated as likely specific DEHA metabolites (see **Fig. 1**) and were obtained by custom synthesis. In addition, a screening was performed with the aim of identifying further specific DEHA metabolites which could be suitable as biomarkers of exposure to DEHA. For that purpose, urine samples from one volunteer (up to 8 h after dose) were analyzed after enzymatic deconjugation. A liquid chromatography-high resolution mass spectrometry screening approach with ESI-Q-Orbitrap mass spectrometry, which was previously applied to the identification of metabolites of the alternative plasticizer DEHTP (Lessmann et al. 2018), proved to be insufficiently sensitive for the detection of the specific secondary oxidized DEHA metabolites. Further tests with analytical standards of 5OH-MEHA, 5oxo-MEHA, and 5cx-MEPA showed that weakly alkaline buffer (NH_4HCO_3 buffer; 5 mM, pH= 8) as eluent, instead of 0.05% acetic acid, improved the sensitivity. Yet, this improvement in sensitivity was insufficient for the detection of the metabolites, other than adipic acid and 5OH-MEHA in the majority of urine samples after the oral dose. Three further suspect screening approaches were applied using LC-ESI-triple-quadrupole-MS/MS without analyte enrichment, as well as with two different approaches for

analyte enrichment and matrix depletion (see section 5.2.5. and the Supplementary Material). In all three approaches, 5OH-MEHA, and 5cx-MEPA were confirmed as urinary metabolites of DEHA. The metabolite 5oxo-MEHA could be confirmed with both approaches using online-SPE-LC-MS/MS (Supplementary Material: **Fig. S1**). Further specific metabolites could not be identified. Preliminary tests also indicated that MEHA was only a minor metabolite of low quantitative relevance compared to the oxidized monoester metabolites. Accordingly, only the three specific, oxidized DEHA metabolites (5OH-MEHA, 5oxo-MEHA, and 5cx-MEPA), were investigated for their formation and urinary excretion and analyzed quantitatively

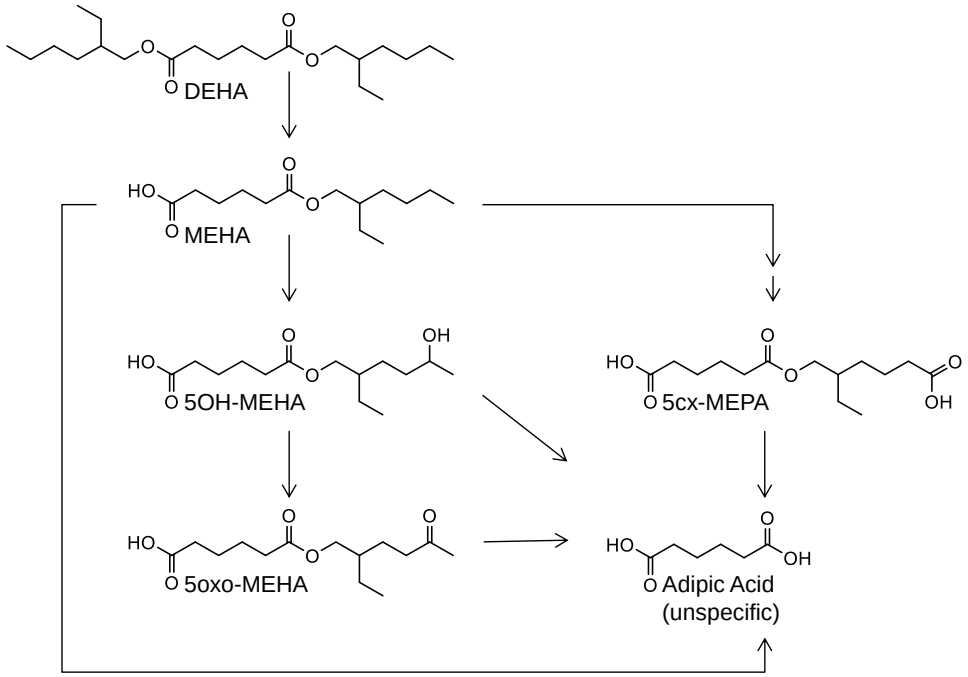

Fig. 1: *Human DEHA metabolism leading to the three specific side chain oxidized metabolites 5OH-MEHA, 5oxo-MEHA and 5cx-MEPA and non-specific adipic acid. For simplification, phase two metabolites (e.g. glucuronic acid conjugates) are not shown*

In addition to the specific side chain oxidized monoester metabolites, adipic acid (AA) was found as urinary DEHA metabolite, confirming previous findings in animal studies of rats (Takahashi et al. 1981). However, it is important to highlight that AA does not represent a specific exposure biomarker for DEHA (Silva et al. 2013; Calafat et al. 2015), as it can be formed by any compound bearing an adipic acid moiety, such as di-*n*-butyl-adipate (DnBA) or diisononyl adipate (DINA). Exogenous sources of AA (AA is permitted as a food additive, e.g., in the EU (European Commission 2012) and the U.S. (U.S. Food and Drug Administration (FDA) 2018)), as well as its endogenous formation (Liebich et al. 1980; Pettersen et al. 1972) further preclude its use as a specific biomarker of DEHA exposure.

5.3.2. Urinary excretion kinetics after oral DEHA dose

Four volunteers donated 21, 22, 23, and 27 individual urine samples with total volumes of 3646, 6668, 2852, and 5797 mL. Each pre- and post-dose urine sample was analyzed for 5cx-MEPA, 5OH-MEHA, and 5oxo-MEHA using our recently published online-SPE-LC-MS/MS method with stable isotope dilution (Nehring et al. 2019). Furthermore, each pre-dose sample and each sample within 24 h post-dose was analyzed for adipic acid by LC-MS/MS (see Supplementary Material). Oxidized monoester metabolites were below the LOQ in all pre-dose urine samples (Supplementary Material: **Fig. S2, a**). In the post-dose urine samples, all three oxidized metabolites rapidly emerged (Supplementary Material: **Fig. S2, b**), with maximum concentrations (c_{max1} and c_{max2} – **Table 1**) being 2-3 (5OH-MEHA and 5cx-MEPA) and up to 2 (5oxo-MEHA) orders of magnitude higher than the LOQ, respectively. Concentrations of AA were above the LOQ in all urine samples. The pre-dose urine samples concentrations were in the mg/L range (0.36-3.35 mg/L; 0.83-1.96 mg/g creatinine; see also **Fig. S3**). Post-dose maximum concentrations (c_{max}) were increased 4- to 5-fold compared to this background

Table 1: *Elimination kinetics for 5cx-MEPA, 5OH-MEHA, and 5oxo-MEHA in four volunteers after a single oral dose. Peak concentrations (c_{max1} and c_{max2}) post-dose and their respective time points (t_{max1} and t_{max2}) are given for both maxima (mean values; ranges in parentheses). Given the limited number of data points in case of 5oxo-MEHA and 5OH-MEHA, as well as between c_{max1} and c_{max2} in case of 5cx-MEPA, elimination half-lives ($t_{1/2}$) were only calculated for the elimination phase following c_{max2} and only for 5cx-MEPA*

	5cx-MEPA	5OH-MEHA	5oxo-MEHA
c_{max1} [µg/g creatinine]	42.5 (23.3-62.8)	17.9 (7.70-41.5)	15.3 (3.65-42.2)
t_{max1} [h]	1.7 (1.5-2.3)	1.7 (1.5-2.3)	1.7 (1.5-2.3)
c_{max2} [µg/g creatinine]	42.7 (26.8-58.1)	10.8 (5.11-14.4)	7.4 (2.30-10.0)
t_{max2} [h]	5.5 (3.8-6.4)	5.4 (4.8-6.3)	5.4 (4.8-6.3)
$t_{1/2}$ [h]	2.9 (2.1-3.8)	-	-

Urinary excretion kinetics for all three oxidized monoester metabolites and all four volunteers are shown in **Fig. 2**. Absolute concentrations (in µg/L, left panel), creatinine-adjusted concentrations (in µg/g creatinine, middle), and the excretion rates (in µg/h, right panel) are shown on a logarithmic scale. For all three specific metabolites two peak concentrations (c_{max1} and c_{max2} – see **Table 1**) were observed, most distinguishable for 5cx-MEPA. For all of these metabolites, c_{max1} was reached between 1.5 and 2.3 h and c_{max2} was reached between 3.8 and 6.4 h. For a more extensive investigations of the elimination kinetics, one volunteer was given a single dose of the monoester metabolite MEHA (equimolar to the DEHA dose). Only the first of the two maxima for urinary concentrations of the specific DEHA metabolites could be observed (c_{max1}= 90 min (5cx-MEPA), 40 min (5OH-MEHA), and 40 min (5oxo-MEHA); see Supplementary Material: **Fig. S4**). This observation is aligned with the hypothesis made for the phthalate plasticizers di(2-ethylhexyl) phthalate (DEHP) and di(2-propylheptyl) phthalate (DPHP) (Kessler et al. 2012; Klein et al. 2018) that partial presystemic hydrolysis of the diester plasticizers is expected to occur in the gastrointestinal tract yielding the monoester, which is then absorbed into the portal blood. Another share of the intact diester is expected to be taken up in a delayed fashion via the lymphatic system. After DEHA dose, concentrations of all three specific metabolites past c_{max2} declined rapidly and the majority of the metabolites was excreted within 24 h (5cx-MEPA, 5OH-MEHA, and 5oxo-MEHA: 98-100% for each metabolite). Elimination kinetics for adipic acid are shown in **Fig. S3** (creatinine-adjusted data only). Even though fluctuating, high background exposures complicated the interpretation of AA elimination kinetics, peak concentrations were observed between 1.5 and 7.6 h post-dose (c_{max}: 3.1-9.7 mg/g creatinine), similar to those of the monoester metabolites.

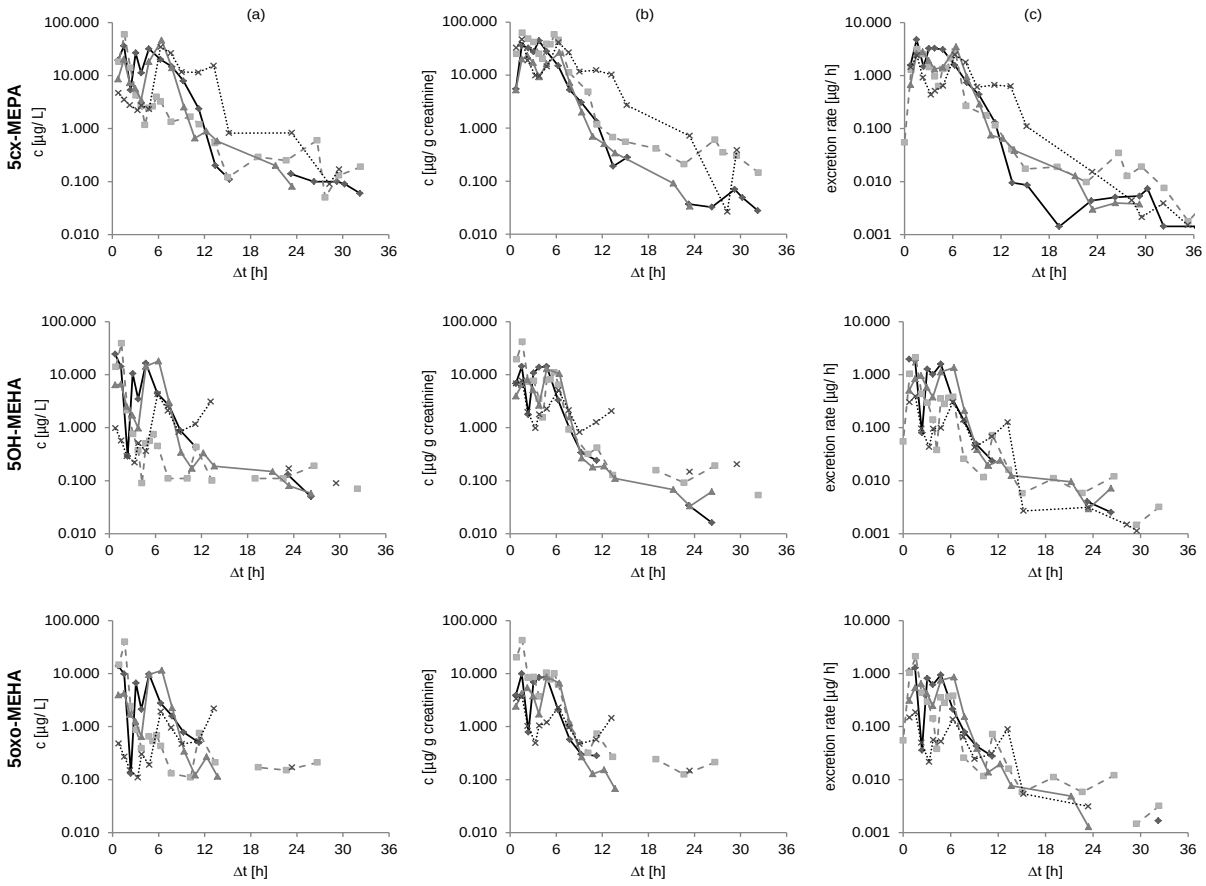

Fig. 2: *Elimination kinetics of 5cx-MEPA (top), 5OH-MEHA (middle), and 5oxo-MEHA (bottom) for all four volunteers after oral DEHA dose. Unadjusted concentrations in µg/L are presented in column (a), creatinine-adjusted concentrations in µg/g creatinine in column (b), and excretion rates in µg/h in column (c)*

5cx-MEPA was the most abundant monoester metabolite in all four volunteers with peak concentrations of 23.3-62.8 µg/g creatinine (c_{max1}) and 26.8-58.1 µg/g creatinine (c_{max2}), respectively. Both, 5oxo-MEHA and 5OH-MEHA concentrations were lower in both maxima (c_{max1}: 3.65-42.2 µg/g creatinine and 7.70-41.5 µg/g creatinine, respectively; c_{max2}: 2.30-10.0 µg/g creatinine and 5.11-14.4 µg/g creatinine, respectively). Both, 5cx-MEPA and 5OH-MEHA were above the LOQ in all post-dose urine samples within 11 h (ranges over all volunteers: 15.3-32.3 h for 5cx-MEPA, and 11.3-26.3 h for 5OH-MEHA), whereas in one volunteer 5oxo-MEHA was above the LOQ only until 3.8 h post-dose (across all volunteers: 3.8-13.7 h). Elimination half-lives $t_{1/2}$ were only calculated after the second peak concentration (past c_{max2}; **Table 1**), as the number of data points was insufficient to appropriately describe the first elimination phase (between c_{max1} and c_{max2}). Furthermore, the number of data points after c_{max2} was insufficient to describe the elimination phase of 5oxo-MEHA and for some volunteers also of 5OH-MEHA. Accordingly, elimination half-lives ($t_{1/2}$) were calculated for 5cx-MEPA only. In case

of the non-specific DEHA metabolite AA, $t_{1/2}$ was not calculated due to high background concentrations with high intra-individual fluctuations and inter-individual differences.

Urinary excretion fractions (F_{UE}) were calculated for the three specific DEHA metabolites, based on the total excreted amounts of each metabolite within 48 h post-dose. In the case of adipic acid, the background concentrations mentioned above allowed only rough estimates of the F_{UE}. For that purpose, median background concentrations were used as a correction of urinary AA concentrations and to calculate the total excreted AA amounts (for a detailed description of the procedure see the Supplementary Material). Calculated (and, in case of AA, estimated) F_{UE}s are shown in **Table 2**. Adipic acid was confirmed as a major (yet non-specific) urinary metabolite in humans with a dose share of approximately 10-40%. The major specific metabolite was 5cx-MEPA, representing 0.20% of the oral dose, with F_{UE}s very consistent between the four volunteers (0.17-0.24%). The metabolites 5OH-MEHA and 5oxo-MEHA each made up a smaller share of the DEHA dose excreted in urine compared to 5cx-MEPA (F_{UE} >2-fold lower; **Table 2**). Thus, the total dose recovery in urine was 40% and less, which is in line with findings by Takahashi et al. (1981) who recovered approximately 40 and 60% of an oral dose of ^{14}C-labeled DEHA administered to rats as respiratory CO_2. Accordingly, it can also be expected that the human metabolic pathway leading to respiratory CO_2 (via total breakdown of adipic acid and potentially also 2-ethylhexanol (Rusoff et al. 1960; Albro 1975)) will strongly contribute to DEHA elimination.

Table 2: Urinary excretion fractions (F_{UE}) of the specific, oxidized monoester DEHA metabolites; 5cx-MEPA, 5OH-MEHA, and 5oxo-MEHA after a single oral dose in four healthy volunteers (mean values; ranges in parentheses). For the hydrolytic metabolite adipic acid, only rough estimates for F_{UE} are provided based on background corrected urinary metabolite levels within 24 h post-dose

	5cx-MEPA	5OH-MEHA	5oxo-MEHA	Σ specific metabolites	adipic acid
0-24 h [%]					
Mean	0.203	0.067	0.047	0.317	10-40
(Range)	(0.164-0.237)	(0.027-0.096)	(0.014-0.060)	(0.237-0.393)	
24-48 h [%]					
Mean	0.001	0.000	0.000	0.001	-[1]
(Range)	(0.000-0.003)	(0.000-0.000)	(0.000-0.000)	(0.000-0.003)	
Total 0-48h [%]					
Mean	**0.20**	**0.07**	**0.05**	**0.32**	
(Range)	**(0.17-0.24)**	**(0.03-0.10)**	**(0.01-0.06)**	**(0.24-0.39)**	**10-40[1]**

[1]only 24 h post-dose urine samples were analyzed for adipic acid

The F_{UE}s for the specific sidechain-oxidized DEHA metabolites were considerably lower in comparison to the corresponding oxidized monoester metabolites of the two structurally related plasticizers DEHP and DEHTP (mean values: 16-25% (5OH-MEHP), 11-15% (5oxo-MEHP), and 14-22% (5cx-MEPP) for DEHP and 1.82% (5OH-MEHTP), 1.01% (5oxo-MEHTP), and 12.95% (5cx-MEPTP) for DEHTP, respectively (Koch et al. 2005; Anderson et al. 2011; Lessmann et al. 2016)). However, the two regioisomeric plasticizers, DEHP and DEHTP, had a 10-fold quantitative difference in F_{UE}s for the 5 hydroxy- and 5 oxo-metabolites, whereas F_{UE}s for the 5 carboxy metabolite were comparable. Furthermore, quantitative differences in metabolism between DEHA and DEHP are in line with comparative *in vitro* investigations, in tissue preparations from liver, pancreas, and small intestine of rats: Takahashi et al. (1981) observed rapid hydrolysis of DEHA to AA, whereas DEHP was hydrolyzed more slowly and hydrolysis stopped at the monoester stage. Moreover, for two UV filters containing a 2-ethylhexyl ester moiety (octocrylene (OC) and 2-ethylhexyl salicylate (EHS)) similarly low or even lower (in case of 5OH-OC: 0.005-0.011%) F_{UE}s were observed for the sidechain-oxidized metabolites (5 hydroxy-, 5 oxo-, 5 carboxy- metabolites) (Bury et al. 2019a; 2019b). For all four substances, the 5OH metabolite is 1.4-2.5-fold more abundant than the 5oxo metabolite. The 5cx metabolite on the other hand is the major specific metabolite in case of DEHTP and (although less pronounced) DEHA, whereas it has approximately the same F_{UE} as the 5OH metabolite in case of DEHP and EHS (Koch et al. 2005; Anderson et al. 2011; Lessmann et al. 2016; Bury et al. 2019a). For DEHA, the ratios between urinary excretion fractions were consistent with metabolite ratios observed in pilot populations from the U.S. American general population (semi-quantitative data for the sum of respective isomers) (Silva et al. 2013) and in pregnant Brazilian women, as well as after a staged DEHA exposure (consumption of food stored in PVC-cling film overnight) in German volunteers (Nehring et al. 2019). In contrast, other research groups reported higher 5oxo-MEHA concentrations compared to 5OH-MEHA in convenience samples (Been et al. 2019; Bastiaensen et al. 2019). This discrepancy with our data and findings by Silva and coworkers might be explained by the use of a surrogate internal standard eluting minutes after the respective DEHA metabolites.

In addition to the total excreted metabolite amounts we investigated the glucuronidation patterns of the three specific DEHA metabolites in one volunteer (34 years of age, 91 kg, male; for further details see the Supplementary Material). The study found that 61% of 5cx-MEPA was excreted in its free, non-conjugated form, while the less polar metabolites 5OH-MEHA and 5oxo-MEHA were excreted without conjugation to less than 10% (8.5% and 6.8%, respectively). This data is consistent with previous studies

on the structurally related alternative plasticizers di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH) and DEHTP: also for these two substances the free (non-glucuronidated) form was more abundant in urine in case of the more polar carboxy metabolite compared to the oxo and hydroxy metabolites (Koch et al. 2013; Lessmann et al. 2016).

5.3.3. Calculation of daily intakes

To estimate daily intakes (DI) values, the mean F_{UE} for 5cx-MEPA (**Table 2**) was used on metabolite concentrations in spot urine samples from two pilot populations, of pregnant Brazilian women (n = 44) and German adults from the general population (n = 32) (Nehring et al. 2019). Considering that 5cx-MEPA was found above the LOQ in less than 50% of the samples in both populations, median Dis could not be calculated. The maximum (95th percentile) Dis based on urinary 5cx-MEPA concentrations were 4.91 (2.69) µg/kg bw/day for Brazilian women and 11.8 (1.56) µg/kg bw/day for German adults. Maximum calculated daily intakes were at least a factor of 25 below the TDI for DEHA (0.3 mg/kg bw/day) and a factor of ≥10 below the doses applied in the oral dosing study. Additionally, the daily intake after a staged DEHA exposure (six volunteers consumed food, which previously was wrapped in DEHA containing PVC-cling film) was calculated (Nehring et al. 2019). The maximum DI of 114 µg/kg bw/day (95th percentile: 109 µg/kg bw/day, 50th percentile: 25.0 µg/kg bw/day) was only a factor of 3 below the TDI for DEHA. This staged DEHA exposure was only a single exposure. Repeated exposures via food can be expected and might result in higher Dis. However, these Dis should be regarded as a proof of concept. Larger populations need to be investigated for representative data.

5.4. Conclusion

This study presents the first quantitative data on human *in vivo* DEHA metabolism and urinary excretion after oral uptake. We newly identified 5cx-MEPA as a specific urinary DEHA metabolite and most abundant specific DEHA exposure biomarker. In comparison to the other two specific side chain oxidized metabolites 5OH-MEHA and 5oxo-MEHA its urinary excretion fraction is approximately 2-fold higher. In agreement with this finding, 5cx-MEPA was the leading DEHA exposure biomarker in two pilot populations from Germany and Brazil with highest detection rates and highest concentration levels. In individuals consuming food that had been wrapped in DEHA containing PVC cling film (Nehring et al.

2019), all three metabolites could be quantified, and concentrations were in line with the metabolite ratios observed in the single oral dose study. Thus, 5cx-MEPA seems best suited to be applied as the most sensitive biomarker of DEHA exposure with 5OH-MEHA and 5oxo-MEHA providing confirmation at higher exposure levels. We also confirmed adipic acid as a major urinary metabolite of DEHA (representing up to 40% of the oral DEHA dose), but hold it unsuitable as a biomarker for DEHA exposure assessment owing to specificity issues. Due to the rapid breakdown of DEHA after oral uptake, the urinary excretion fractions (F_{ue}) of the specific, side chain oxidized metabolites were rather small (0.20% for 5cx-MEPA). However, the F_{ue} of 5cx-MEPA was very consistent between the four volunteers (0.17-0.24%) and can thus be applied for the robust and specific calculation of DEHA intakes. For that purpose, our previously described analytical method in conjunction with the toxicokinetic data presented here provide valuable tools for a rugged exposure and risk assessment for DEHA.

5.5. Acknowledgement

The development of the analytical method and its application in investigating human metabolism and population samples are part of a large-scale 10-year project on the advancement of human biomonitoring in Germany. This project is a cooperation agreed in 2010 between the Federal Ministry for the Environment, Nature Conservation, and Nuclear Safety (BMU) and the Verband der chemischen Industrie e.V. (German Chemical Industry Association– VCI) and is managed by the German Environment Agency (UBA). Experts from governmental scientific authorities, industry and science accompany the project in substance selection and method development (Kolossa-Gehring et al. 2017). The analytical method development was financed by the Chemie Wirtschaftsförderungsgesellschaft mbH. We would like to thank Anderson Joel Martino-Andrade who provided the samples from pregnant Brazilian women.

5.6. Compliance with ethical standards

5.6.1. Ethical approval

The study has been approved by the ethical review board of the medical faculty of the Ruhr University Bochum, Germany (IRB Reg. No.: 15-5422).The study was performed in accordance with the Code of Ethics of the World Medical Association (1964 Declaration of Helsinki and its later amendments). The study design was presented to the participants in written form and written informed consent was obtained from each participant.

5.6.2. Conflict of interest

The participation of Rainer Otter as co-author was conducted as part of his employment responsibilities with BASF SE, a manufacturer of DEHA, and his advisory role within the BMU-VCI cooperation project on human biomonitoring. The interpretation and views expressed in this manuscript are not necessarily those of the co-author´s employer.

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Supplementary Material

This supplementary material includes:

Further information on the applied DEHA metabolite screening approaches (instrument setup, LC-MS/MS conditions, putative metabolites screened for, and excretion kinetics for tentative metabolites), chromatogram of a pre-dose and post-dose urine sample analyzed for the oxidized metabolites, quantification of adipic acid, elimination kinetics for adipic acid, elimination kinetics after equimolar MEHA dose, and glucuronidation patterns of specific DEHA metabolites.

S5.1. Metabolite screening:

Urine samples were treated as described in Nehring et al. (2019), however, without addition of internal standards. The Agilent Technologies LC 1200 system (Waldbronn, Germany) used consisted of a G1329A autosampler, a G1311A quaternary pump coupled with a G4225A vacuum degasser, a G1312A binary pump coupled with a G1322A vacuum degasser and a G1316A thermostated column compartment with 6-port switching valve. A phenylalkyl-modified silica gel column (Accucore Phenyl-X 150 × 3 mm, particle size 2.6 µm with corresponding guard column; Thermo Scientific, Franklin, MA, USA) was used for chromatographic separation with a flow rate of 300 µL in all three screening approaches. The gradients and turbulent flow chromatography columns (where applicable) are listed in the respective subsection for each approach:

S5.1.1. LC-MS/MS approach

5 µL of a processed sample were analyzed using the solvent gradient shown in **Table S1**. Eluents were water (solvent A) and acetonitrile (solvent B), both containing 0.05% acetic acid.

Table S1: *Solvent gradient for chromatographic separation for the LC-MS/MS approach*

Time [min]	A [%]	B [%]
0	95	5
45	5	95
50	5	95
50.1	95	5
60	95	5

S5.1.2. Online-SPE-LC-MS/MS approach with two TFC columns

The instrument setup was as described in Nehring et al. (2019), except two turbulent flow chromatography (TFC) columns were used in line for analyte enrichment and sample clean-up (TurboFlow Phenyl 50 x 0.5 mm; TurboFlow Cyclone-P 50 x 0.5 mm; Thermo Scientific, Franklin, MA, USA). This setup was an adaption of the metabolite screening approach applied for the UV filters octocrylene and 2-ethylhexyl salicylate (Bury et al. 2019a; 2019b). 50 µL of processed urine sample were injected. After 3 minutes of flushing for matrix depletion, the 6-port valve was switched into transfer position and kept in this position for the whole chromatographic run. The solvent gradient used for analyte enrichment is shown in **Table S2**, while the solvent gradient for chromatographic separation is shown in **Table S3**.

Table S2*: Solvent gradient for matrix depletion for the two TFC column sample clean-up approach*

Time [min]	Flow rate [µL/min]	A [%]	B [%]
0	1500	100	0
4	1500	100	0
5	1500	100	0
12	1000	0	100
14	500	0	100
22	100	100	0
23	100	100	0
24	500	100	0
25	1000	100	0
27	1500	100	0

Table S3: *Solvent gradient for chromatographic separation for the two TFC column sample clean-up approach*

Time [min]	A [%]	B [%]
0	70	30
2.5	70	30
4	60	40
19	45	55
20	5	95
22	5	95
23	70	30
27	70	30

S5.1.3. Online-SPE-LC-MS/MS approach using a mixed mode TFC column

The instrument setup again was as described in Nehring et al. (2019), except a mixed mode (reversed phase/strong anion exchange) TFC column was used for analyte enrichment and sample clean-up (TurboFlow Cyclone MAX 50 x 0.5 mm; Thermo Scientific, Franklin, MA, USA). The injection volume was again 50 µL. After 3 minutes of flushing for matrix depletion, the analytes were transferred to the analytical column; the 6-port valve was switched into transfer position and kept in this position until 43 min runtime. Eluents were water (solvent A) and acetonitrile (solvent B), both containing 0.05% acetic

acid, as well as NH_4HCO_3 buffer (solvent C; 5 mM, pH= 8). The solvent gradient used for analyte enrichment is shown in **Table S4**; the solvent gradient for chromatographic separation is shown in **Table S5**.

Table S4: *Solvent gradient for analyte enrichment using the mixed mode TFC column*

Time [min]	Flow Rate [µl/min]	A [%]	B [%]	C [%]
0	1500	0	0	100
4	1500	0	0	100
5	1000	100	0	0
6	500	100	0	0
7	100	100	0	0
12	100	100	0	0
12.5	100	5	95	0
15	100	5	95	0
16	100	100	0	0
25	100	100	0	0
43	500	0	0	100
44	1000	0	0	100
45	1500	0	0	100
48	1500	0	0	100

Table S5: *Solvent gradient for chromatographic separation using the mixed mode TFC column*

Time [min]	A [%]	B [%]
0	95	5
1.5	95	5
5	95	5
7	85	15
15	85	15
35	5	95
37	5	95
37.1	95	5
48	95	5

S5.1.4. Mass spectrometric conditions

Detection in all three approaches was performed by ESI-MS/MS using an AB Sciex 5500 QTrap mass spectrometer (Darmstadt, Germany). The MS instrument gases (nitrogen) were set as follows: curtain gas 25 psi, nebulizer and heater gas 45 psi and 50 psi, and collision gas pressure was set to 'medium' setting. The source heater temperature was 550 °C and the ion spray voltage was -4.5 kV. Entrance and collision cell exit potentials were -10 V and -11 V. For each postulated metabolite, (if possible) at least two mass transitions were analyzed. Fragment ions and MS parameters (declustering potentials and collision energies – see **Table S6**) were chosen in analogy to the known fragmentation behavior of 5OH-MEHA, 5oxo-MEHA, and 5cx-MEPA (Nehring et al. 2019). The fragments at *m/z* 127, *m/z* 101,

and *m/z* 83 had been observed in all these three metabolites. Accordingly, mass transitions in the screening experiments were based on these fragment ions or their respective chemically modified (e.g., hydroxylated) analogs. The formation of *m/z* 127 can be explained by elimination of water from free adipic acid (observed as a fragment ion at *m/z* 145). For 5cx-MEPA, *m/z* 127 could also be formed by elimination of water from the adipic acid moiety and subsequent ester cleavage. Elimination of CO_2 from free adipic acid results in *m/z* 101 and elimination of CO_2 from *m/z* 127 results in *m/z* 83. In addition, a fragment at *m/z* 159 was observed for 5cx-MEPA and can be explained as the alkyl side chain fragment of 5cx-MEPA (5-(hydroxymethyl)heptanoic acid). This fragment was considered for tentative metabolites bearing a carboxylic acid moiety in the modified 2-ethylhexyl side chain. Putative metabolites are named using the code [basic structure]-[modification 1]/[modification 2]…/[modification n] with the following modifications: OH: hydroxylation, oxo: further oxidation to carbonyl group, cx: further oxidation to carboxylic acid group, *β*-Ox: *β*-oxidation (i.e., elimination of a C_2H_4 unit from a linear aliphatic carboxylic acid moiety), SO_4: hydroxylation followed by sulfatation, Gly: conjugation of a carboxylic acid group with glycine, Gln: conjugation of a carboxylic acid group with glutamine. Given the high number of mass transitions (43 metabolites; total of 89 transitions), the screening was divided into three separate LC-MS/MS experiments (mass transitions evenly distributed across the three experiments) for each approach, resulting in scan rates of ≥1 Hz for each mass transition.

Table S6: *Mass transitions and corresponding MS/MS parameters of each putative DEHA metabolite.*

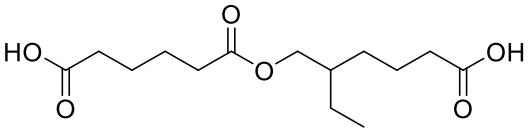
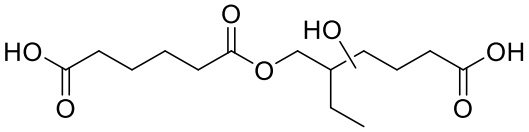
	Putative metabolite	Precursor ion [*m/z*]	Declustering potential [V]	Product ion [*m/z*]	Collision energy [eV]
1	[MEHA]-[cx][1]	287	-87	127*	-15
				83*	-23
				159*	-22
2	[MEHA]-[cx]/[OH][1]	303	-80	127	-15
				83	-23
				175 (corr. To *m/z* 159*)	-22

Table S6 – continued: *Mass transitions and corresponding MS/MS parameters of each putative DEHA metabolite*

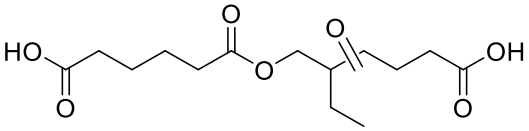
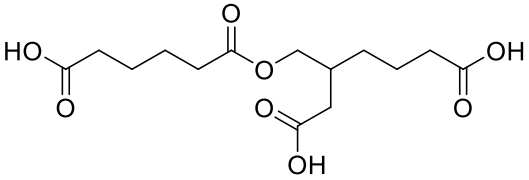
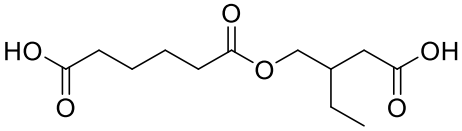
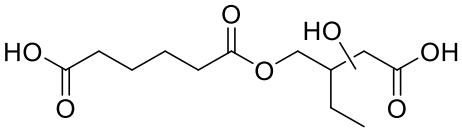
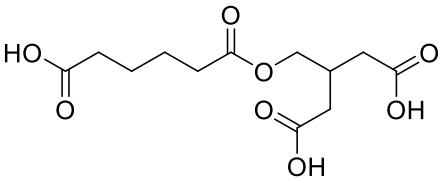
	Putative metabolite	Precursor ion [*m/z*]	Declustering potential [V]	Product ion [*m/z*]	Collision energy [eV]
3	[MEHA]-[cx]/[oxo][1] 	301	-80	127 83 173 (corr. To *m/z* 159*)	-15 -23 -22
4	[MEHA]-[cx]/[cx] 	317	-70	127 83 189 (corr. To *m/z* 159*)	-15 -23 -22
5	[MEHA]-[cx]/[*β*-Ox] 	259	-70	127 83 131 (corr. To *m/z* 159*)	-15 -23 -22
6	[MEHA]-[cx]/[*β*-Ox]/[OH] 	275	-70	127 83 147 (corr. To *m/z* 159*)	-15 -23 -22
7	[MEHA]-[cx]/[*β*-Ox]/[cx] 	289	-60	127 83 161 (corr. To *m/z* 159*)	-15 -23 -22

Table S6 – continued: *Mass transitions and corresponding MS/MS parameters of each putative DEHA metabolite*

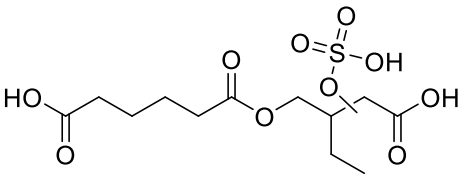
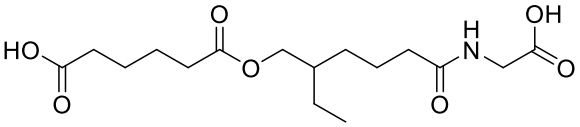
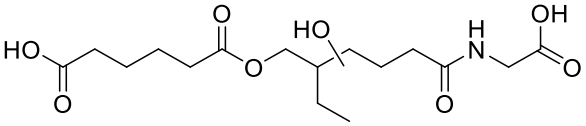
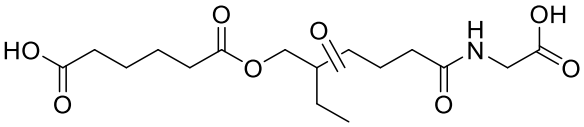
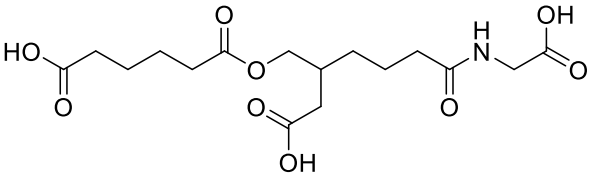
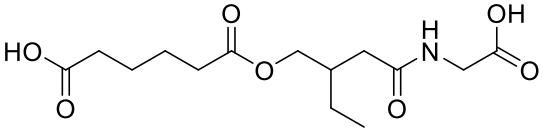
	Putative metabolite	Precursor ion [*m/z*]	Declustering potential [V]	Product ion [*m/z*]	Collision energy [eV]
8	[MEHA]-[cx]/[*β*-Ox]/[SO_4]	355	-70	127	-15
				83	-23
9	[MEHA]-[cx]/[Gly][1]	344	-70	127	-15
				83	-23
10	[MEHA]-[cx]/[Gly]/[OH][1]	360	-70	127	-15
				83	-23
11	[MEHA]-[cx]/[Gly]/[oxo][1]	358	-70	127	-15
				83	-23
12	[MEHA]-[cx]/[Gly]/[cx][1]	374	-70	127	-15
				83	-23
13	[MEHA]-[cx]/[*β*-Ox]/[Gly]	316	-70	127	-15
				83	-23

Table S6 – continued: *Mass transitions and corresponding MS/MS parameters of each putative DEHA metabolite*

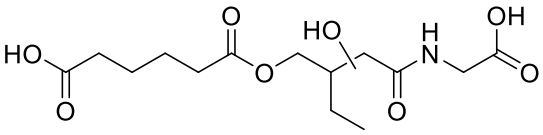
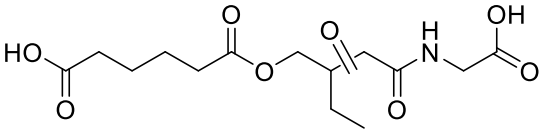
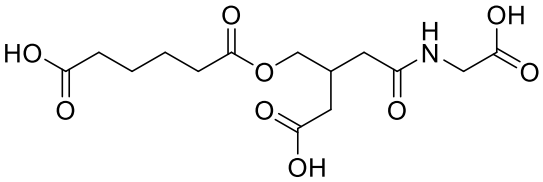
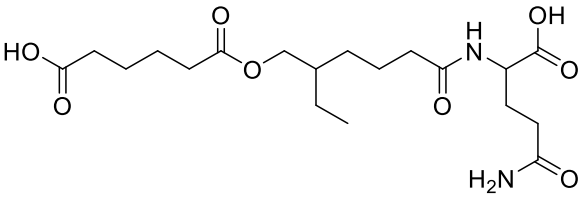
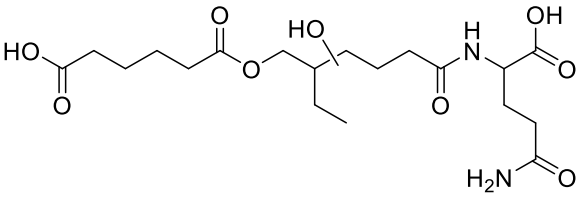
	Putative metabolite	Precursor ion [*m/z*]	Declustering potential [V]	Product ion [*m/z*]	Collision energy [eV]
14	[MEHA]-[cx]/[*β*-Ox]/[Gly]/[OH]	332	-70	127	-15
				83	-23
15	[MEHA]-[cx]/[*β*-Ox]/[Gly]/[oxo]	330	-70	127	-15
				83	-23
16	[MEHA]-[cx]/[*β*-Ox]/[Gly]/[cx]	346	-70	127	-15
				83	-23
17	[MEHA]-[cx]/[Gln][1]	415	-70	127	-15
				83	-23
18	[MEHA]-[cx]/[Gln]/[OH][1]	431	-70	127	-15
				83	-23

Table S6 – continued: *Mass transitions and corresponding MS/MS parameters of each putative DEHA metabolite*

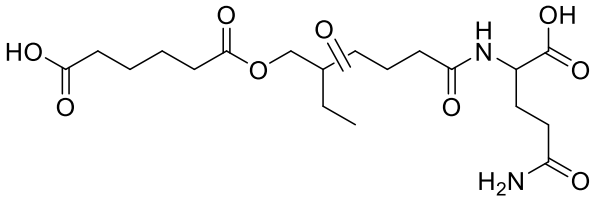
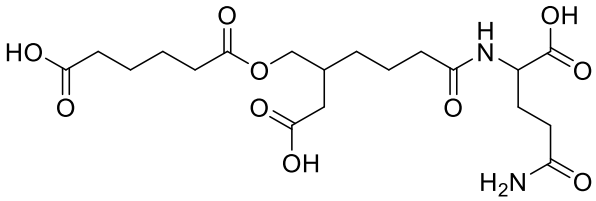
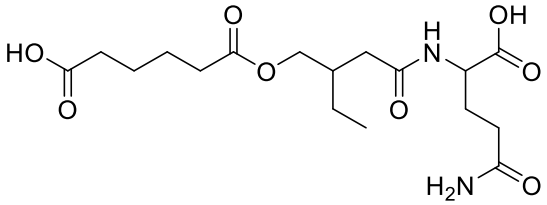
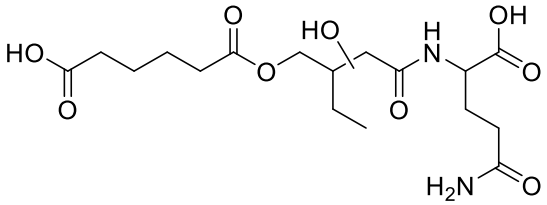
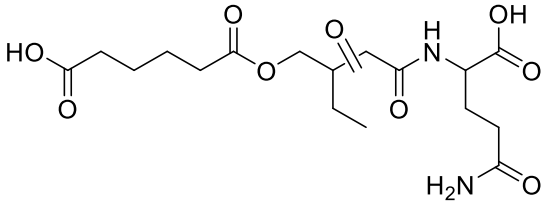
	Putative metabolite	Precursor ion [*m/z*]	Declustering potential [V]	Product ion [*m/z*]	Collision energy [eV]
19	[MEHA]-[cx]/[Gln]/[oxo][1]	429	-70	127	-15
				83	-23
20	[MEHA]-[cx]/[Gln]/[cx][1]	445	-70	127	-15
				83	-23
21	[MEHA]-[cx]/[*β*-Ox]/[Gln]	387	-70	127	-15
				83	-23
22	[MEHA]-[cx]/[*β*-Ox]/[Gln]/[OH]	403	-70	127	-15
				83	-23
23	[MEHA]-[cx]/[*β*-Ox]/[Gln]/[oxo]	401	-70	127	-15
				83	-23

Table S6 – continued: *Mass transitions and corresponding MS/MS parameters of each putative DEHA metabolite*

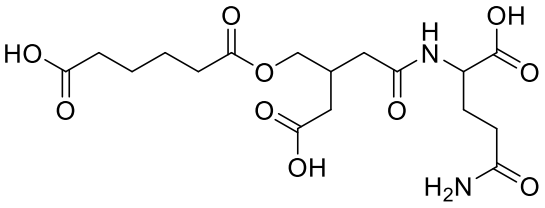
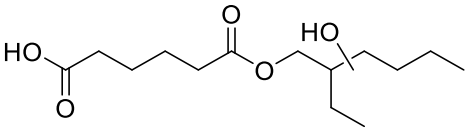
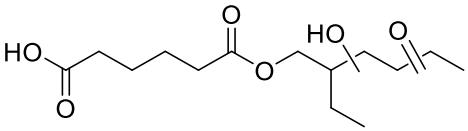
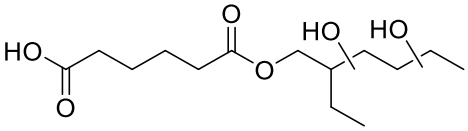
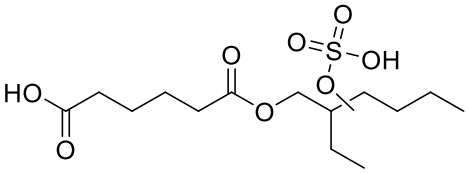
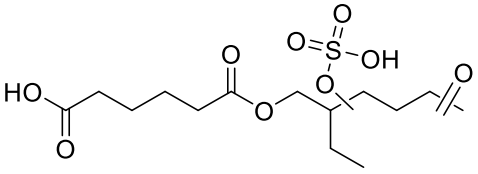
	Putative metabolite	Precursor ion [*m/z*]	Declustering potential [V]	Product ion [*m/z*]	Collision energy [eV]
24	[MEHA]-[cx]/[*β*-Ox]/[Gln]/[cx]	417	-70	127	-15
				83	-23
25	[MEHA]-[OH]	273	-98	127	-15
				83	-23
26	[MEHA]-[OH]/[oxo]	287	-80	127	-15
				83	-23
27	[MEHA]-[OH]/[OH]	289	-80	127	-15
				83	-23
28	[MEHA]-[SO_4]	353	-80	127	-15
				83	-23
29	[MEHA]-[SO_4]/[oxo]	367	-80	127	-15
				83	-23

Table S6 – continued: *Mass transitions and corresponding MS/MS parameters of each putative DEHA metabolite*

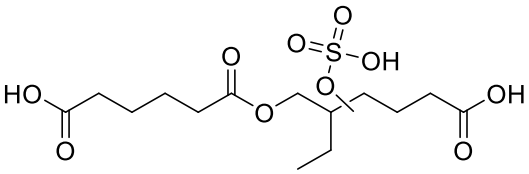
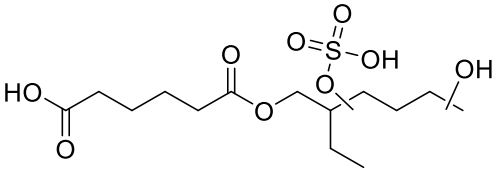
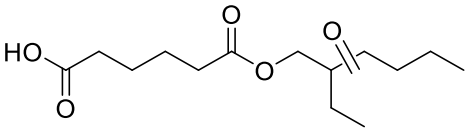
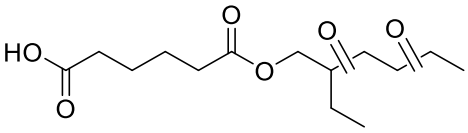
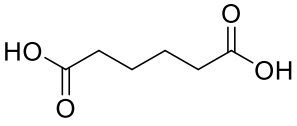
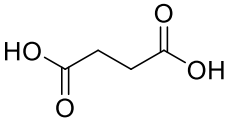
	Putative metabolite	Precursor ion [*m/z*]	Declustering potential [V]	Product ion [*m/z*]	Collision energy [eV]
30	[MEHA]-[SO_4]/[cx][1]	383	-80	127	-15
				83	-23
31	[MEHA]-[SO_4]/[OH]	369	-80	127	-15
				83	-23
32	[MEHA]-[oxo]	271	-11	127	-15
				83	-23
33	[MEHA]-[oxo]/[oxo]	285	-11	127	-15
				83	-23
34	adipic acid	145	-60	101*	-33
				83	-23
35	succinic acid	117	-55	99 (corr. To *m/z* 127*)	-33
				55 (corr. To *m/z* 83*)	-23

Table S6 – continued: *Mass transitions and corresponding MS/MS parameters of each putative DEHA metabolite*

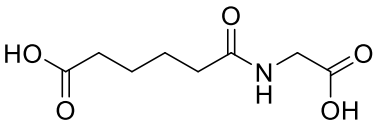
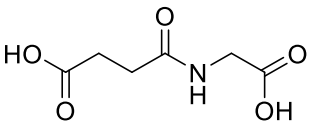
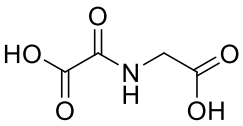
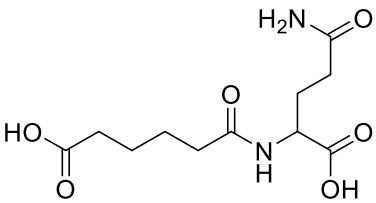
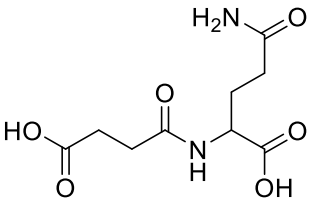
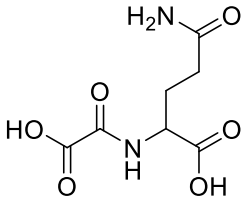
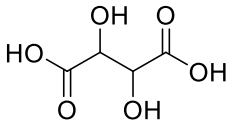
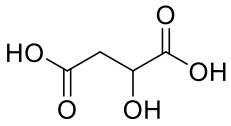
	Putative metabolite	Precursor ion [*m/z*]	Declustering potential [V]	Product ion [*m/z*]	Collision energy [eV]
36	adipic acid-[Gly]	202	-60	144 (corr. To *m/z* 145*)	-24
				83	-23
37	succinic acid-[Gly]	174	-55	116 (corr. To *m/z* 145*)	-24
				98 (corr. To *m/z* 127*)	-23
38	oxalic acid-[Gly]	146	-24	87 (corr. To *m/z* 145*)	-24
					
39	adipic acid-[Gln]	273	-60	144 (corr. To *m/z* 145*)	-24
				83	-23
40	succinic acid-[Gln]	245	-55	116 (corr. To *m/z* 145*)	-24
				98 (corr. To *m/z* 127*)	-23

Table S6 – continued: *Mass transitions and corresponding MS/MS parameters of each putative DEHA metabolite*

	Putative metabolite	Precursor ion [*m/z*]	Declustering potential [V]	Product ion [*m/z*]	Collision energy [eV]
41	oxalic acid-[Gln] 	217	-50	87 (corr. To *m/z* 145*)	-24
42	tartaric acid 	149	-50	87 (corr. To *m/z* 101*)	-23
43	malic acid 	133	-50	115 (corr. To *m/z* 127*)	-23

*: reported for 5OH-MEHA, 5oxo-MEHA, and/or 5cx-MEPA in Nehring et al. (2019).

[1]only one regioisomer shown for simplification

S5.1.5. Results for the identified tentative DEHA metabolites

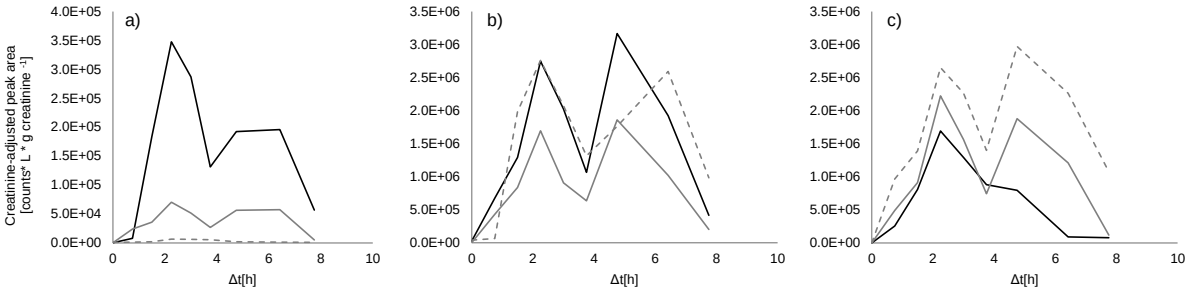


Fig. S1: *Exemplary screening data (creatinine-adjusted peak area plotted against the time) for 5cx-MEPA (m/z 287→83; black solid lines), 5OH-MEHA (m/z 273→83; gray solid lines), and 5oxo-MEHA (m/z 271→83, gray dotted lines) using the following screening approaches: a) LC-MS/MS, b) online-SPE-LC-MS/MS with two TFC columns, and c) online-SPE-LC-MS/MS using a mixed mode TFC column*

S5.2. Chromatogram of a pre-dose and post-dose urine sample analyzed for the oxidized metabolites

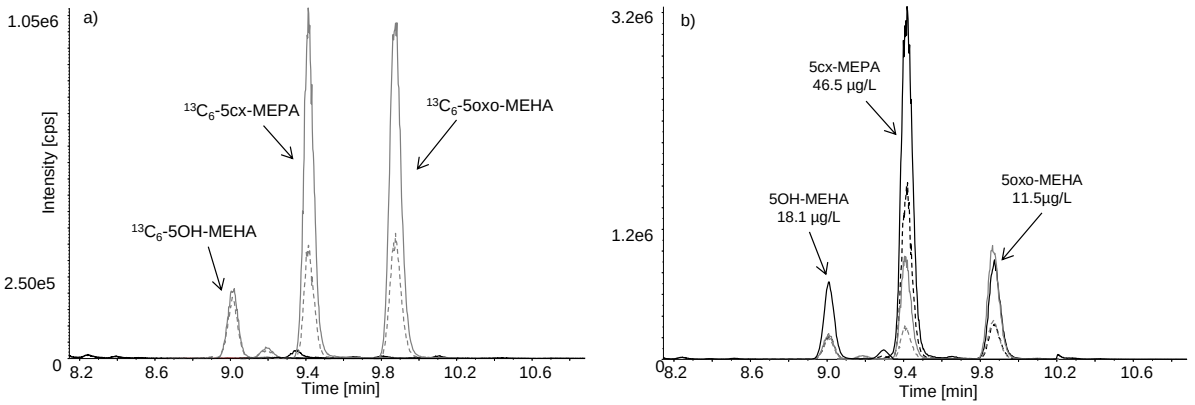


Fig. S2: *Exemplary chromatograms of a pre-dose urine sample (a), and a urine sample 7 h post-dose (b). Non-labeled metabolites in black and internal standards in gray; quantifier transitions as continuous lines and qualifier transitions as dotted lines*

S5.3. Quantification of adipic acid

For chemical analysis, a 1260 Infinity HPLC system (Agilent Technologies; Waldbronn, Germany), consisting a G7111B quaternary pump, a G7167A multisampler and a G7116A thermostated column compartment (kept at 25±0.8 °C), was used. Detection was performed on a 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with electrospray ionization (ESI) in negative ion mode. For chromatographic separation, a C18 reversed phase column with superficially porous particles (Kinetex C18 150 x 3.0 mm, particle size 2.6 µm, with corresponding SecurityGuard; Phenomenex, Aschaffenburg, Germany) was used. Eluents were water (A) and acetonitrile (B), both containing 0.05% acetic acid (see **Table S7** for solvent gradient) with a flow rate of 300 µL/min. The gradient delay volume according to the manufacturer´s specifications was 865 to 1165 µL. The injection volume was 5 µL and the injection process included a needle wash with MeOH/H_2O 8/2 (v/v) for 3 s.

Table S7: *Solvent gradient for adipic acid analysis*

Time [min]	A [%]	B [%]
0	95	5
2	95	5
11	5	95
16	5	95
16.5	95	5
24.5	95	5

The MS instrument gases (nitrogen) were set as follows: curtain gas 35 psi, nebulizer and heater gas 50 psi, and collision gas 8 arbitrary units. The source heater temperature was 500 °C and the ion spray voltage was -4.5 kV. Declustering, entrance, and collision cell exit potentials were -60 V, -10 V, and -10 V. The mass transition *m/z* 145 → 83 (collision energy: -18 eV) was used for quantification and *m/z* 145 → 81 (collision energy: -27 eV) was used as qualifier transition. For the $^{13}C_6$-labeled internal standard, the corresponding mass transitions were used: *m/z* 151 → 88 (quantifier, collision energy: -19 eV) and *m/z* 151→ 86 (qualifier, collision energy: -28 eV).

Stock solutions (1 g/L) of $^{13}C_6$-labeled and non-labeled adipic acid (AA) were prepared in methanol. For an internal standard solution of 200 µg/L $^{13}C_6$-adipic acid in water, a 10-fold diluted solution (0.1 g/L) of the stock solution was prepared in methanol and subsequently diluted with water. For non-labeled AA, two dilutions (100 mg/L and 10 mg/L) were prepared in methanol. These dilutions were used to prepare calibration solutions (concentrations ranging between 1 µg/L and 200 µg/L) in water. The calibration solutions were treated the same way as the diluted urine samples (section 5.2.4 in the manuscript).

Two pooled urine samples (approximately 2 mg/L and 5 mg/L adipic acid) were prepared using urines from the DEHA dosing study. These samples were analyzed in each analytical batch (for quality control and interday precision), as well as eight times in one batch (for within-series precision). For this purpose, these pooled urine samples were treated the same way as the study samples, including 100-fold dilution. The imprecision (coefficient of variation) was ≤ 5%, both within-series (n = 8) and interday (n = 7). To investigate the accuracy of the method, five urine samples with varying creatinine concentrations (0.3 g/L to 2.3 g/L) were analyzed diluted 100-fold and either spiked (at two different levels: 20 µg/L and 160 µg/L) or without addition of AA (for the determination of background AA levels). Relative recoveries after subtraction of background AA levels ranged from 84% to 106%. Based on a signal-to-noise ratio of 10 in matrix, the limit of quantification (LOQ) in 100-fold diluted urine was 1 µg/L, which corresponds to 100 µg/L in the non-diluted urine sample.

S5.3.1. Elimination kinetics and F_{UE} for adipic acid

Strongly fluctuating background concentrations (most evident for volunteers 2 and 4 after 11 h post-dose) of adipic acid, as well as in part high pre-dose levels (compared to the peak concentrations) were observed with strong inter-individual differences (**Fig. S3**). Accordingly, the estimation of F_{UE} was performed after subtraction of individual AA background levels. Background subtraction was performed as previously described for the UV filters octocrylene and 2-ethylhexyl salicylate (Bury et al. 2019a; 2019b) with exception of using individual median values for AA background concentrations. These median background concentrations were individually calculated for each volunteer as the median of urinary AA concentrations in all urine samples after 11 h post-dose.

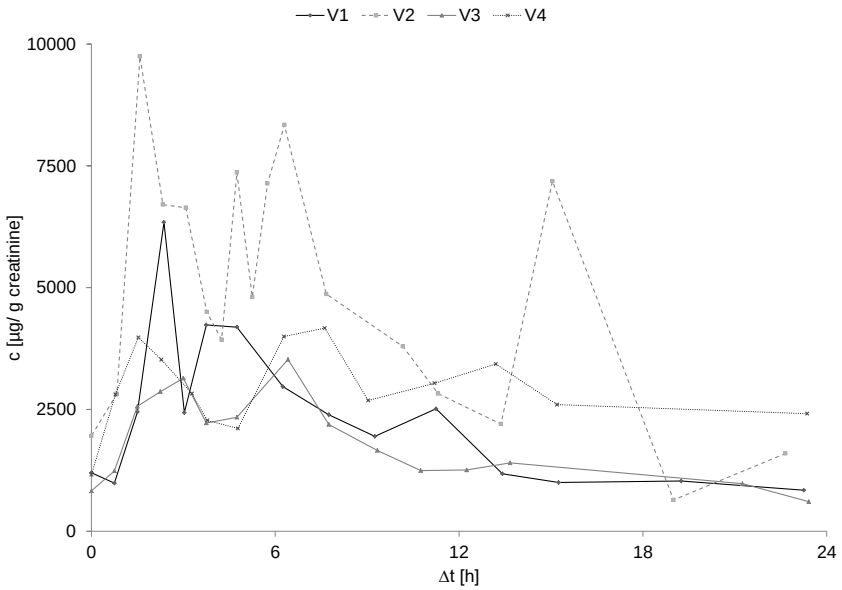


Fig. S3: *Urinary excretion kinetics in µg/g creatinine for adipic acid after oral dose (n = 4)*

S5.4. Oral MEHA dose equimolar to DEHA in one volunteer

All urine samples after MEHA dose were analyzed for the three specific DEHA metabolites. In contrast to the excretion kinetics after DEHA dose, only one single peak concentration was observed for each specific DEHA metabolite after MEHA dose (**Fig. S4**). The second peak concentration observed only after DEHA dose might be explained by partial delayed absorption of intact DEHA via the lymphatic system, as suggested for di(2-ethylhexyl) phthalate (DEHP) and di(2-propylheptyl) phthalate (DPHP) by Kessler et al. (2012) and Klein et al. (2018), and subsequent metabolite formation.

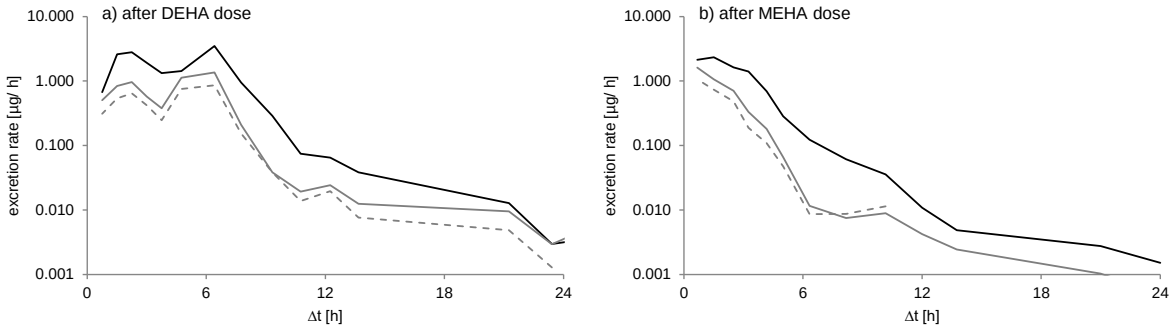


Fig. S4: Excretion rates (in µg/h) for 5cx-MEPA (black solid lines), 5OH-MEHA (gray solid lines), and 5oxo-MEHA (gray dotted lines) after DEHA dose (a), and after MEHA dose (b) in the same volunteer

S5.5. Glucuronidation patterns of 5OH-MEHA, 5oxo-MEHA, and 5cx-MEPA

Individual urine samples from one volunteer (34 years, 91 kg, male) were analyzed. The total amounts (sum of glucuronidated and non-conjugated metabolite) of oxidized monoester metabolites were determined as described in section 5.2.3 of the manuscript. For the determination of free metabolites, the same procedure was applied, however without the addition of β-glucuronidase (replaced by water LC-MS grade). The percentages of non-conjugated ('free') metabolites were calculated in relation to the total amounts (i.e., (total-free)/total).

S5.6. References

Bury D, Griem P, Wildemann T, Brüning T, Koch HM (2019a) Urinary metabolites of the UV filter 2-Ethylhexyl salicylate as biomarkers of exposure in humans. Toxicol. Lett. 309:35–41. doi: 10.1016/j.toxlet.2019.04.001

Bury D, Modick-Biermann H, Leibold E, Brüning T, Koch HM (2019b) Urinary metabolites of the UV filter octocrylene in humans as biomarkers of exposure. Archives of toxicology. doi: 10.1007/s00204-019-02408-7

Kessler W, Numtip W, Völkel W, Seckin E, Csanády GA, Pütz C, Klein D, Fromme H, Filser JG (2012) Kinetics of di(2-ethylhexyl) phthalate (DEHP) and mono(2-ethylhexyl) phthalate in blood and of DEHP metabolites in urine of male volunteers after single ingestion of ring-deuterated DEHP. Toxicology and Applied Pharmacology 264(2):284–291. doi: 10.1016/j.taap.2012.08.009

Klein D, Kessler W, Pütz C, Semder B, Kirchinger W, Langsch A, Gries W, Otter R, Gallien AKE, Wurzenberger X, Filser JG (2018) Single ingestion of di-(2-propylheptyl) phthalate (DPHP) by male volunteers: DPHP in blood and its metabolites in blood and urine. Toxicol. Lett. 294:105–115. doi: 10.1016/j.toxlet.2018.05.010.

Nehring A, Bury D, Kling H-W, Weiss T, Brüning T, Koch HM (2019) Determination of human urinary metabolites of the plasticizer di(2-ethylhexyl) adipate (DEHA) by online-SPE-HPLC-MS/MS. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1124:239–246. doi: 10.1016/j.jchromb.2019.06.019.

Kapitel 6

Quantitative investigation of the urinary excretion of three specific monoester metabolites of the plasticizer diisononyl adipate (DINA)

Gotthardt A, Bury D, Kling H-W, Otter R, Weiss T, Brüning T, Koch HM (2021) Quantitative investigation of the urinary excretion of three specific monoester metabolites of the plasticizer diisononyl adipate (DINA). EXCLI Journal 20:412-425. doi: 10.17179/excli2021-3360

Abstract

Diisononyl adipate (DINA) is a plasticizer used in PVC products as an alternative for restricted phthalate plasticizers. With this study, we provide first data on human DINA metabolism and excretion. We postulated mono(hydoxy-isononyl) adipate (OH-MINA), mono(oxo-isononyl) adipate (oxo-MINA), and mono (carboxy-isooctyl) adipate (cx-MIOA) as specific DINA metabolites based on the known human metabolism of structurally similar adipates and phthalates. Urinary excretion was quantitatively investigated after a single oral dose (113 to145 µg/kg bodyweight) to three healthy volunteers using a newly developed online-SPE-LC-MS/MS method with isotope dilution and LOQs between 0.3 – 0.6 µg/L. OH-MINA turned out to be the major of the three metabolites with consistent urinary excretion fractions (F_{UE}s) of 0.020-0.023% among all volunteers. Oxo-MINA and cx-MIOA were excreted with lower shares (mean: 0.003% and 0.009%, respectively). For all three metabolites, urinary concentrations peaked quickly between 1.4 and 2.3 h post dose with maximum concentrations of 23.1 (OH-MINA), 2.87 (oxo-MINA) and 9.83 µg/L (cx-MIOA). Thus, F_{UE}s and urinary concentrations were rather low for these specific metabolites, with the major share of the dose presumably being excreted as non-specific metabolites such as adipic acid. In a pilot population (n=35) of German adults without known DINA exposure, we could not detect any of the three metabolites, contrary to the dosage study, indicating to population exposures lower than 50 µg/kg bodyweight/day. The new HBM method in conjunction with the new F_{UE}s can be used for objective DINA exposure and risk assessment especially in populations with potentially higher DINA exposures.

6.1. Introduction

Due to their endocrine disrupting potency and reproductive toxicity the use of some ortho-phthalate plasticizers such as di(2-ethylhexyl)phthalate (DEHP) has been strictly regulated in many parts of the world (EC, 1999, 2011a, b, 2018; EPC, 2005, 2006, 2009; CPSC, 2008; Minister of Justice Canada, 2010, 2016). Diisononyl adipate (DINA, CAS registry no. 33703-08-1; EC no. 251-646-7) is an alternative to regulated, high molecular weight phthalates, that is mainly used to achieve low-temperature flexibility in PVC products (BASF SE, 2019; ExxonMobil Chemical, 2016). Consumer near applications include plastic and rubber articles, electrical and electronic products, e.g., wire and cable jacketing, as well as toys and childcare products (Abe et al., 2003, 2012; Biedermann-Brem et al., 2008; Maag et al., 2010; IHS Markit, 2018; ExxonMobil Chemical, 2016; US EPA, 2018; ECHA, 2020a). A further application of DINA is as an additive in greases and lubricants (ECHA, 2020a). DINA may not be used in food contact materials (FCM) in the EU, but in the U.S and parts of Asia it is permitted to be used in FCM in contact with non-fatty and non-alcoholic foods (Malveda et al., 2015; NHFPC China, 2016; FDA, 2019; EC, 2011a).

DINA is produced by esterification of adipic acid with low branched C9-isononanols (BASF SE, 2019) or with a mixture of branched C8-C10 alcohols, containing predominantly C9 alcohols (ExxonMobil Chemical, 2016). Regardless of the manufacturing process (and effectively a different chemical composition), the resulting plasticizer DINA is characterized by the same CAS registry number. The U.S. Environmental Protection Agency refers to DINA as a high production volume chemical (U.S. EPA, 2018). In 2019, 4,500 tons of DINA were consumed in the U.S., while in Western Europe the consumption amounted to 6,000 tons (IHS Markit, 2018).

Scientific data on the toxicity of DINA is limited and no studies regarding reproductive, developmental, and chronic toxicity are publicly available to our knowledge. The European Chemicals Agency (ECHA), the U.S. EPA, and U.S. Consumer Product Safety Commission (CPSC) reported secondary literature describing subchronic oral dose studies in rats and beagles. In rats even at the highest dose level of 500 mg/kg bodyweight (bw)/day (d) no adverse effects could be observed, while in beagles a no observed adverse effect level (NOAEL) of 274 mg/kg bw/d was identified based on decreased body weights and changes in livers and kidneys (U.S. EPA, 2018; ECHA, 2020b, 2020c; Carlisle et al., 2019). So far, a tolerable daily intake (TDI) for DINA has not been derived. The REACH registration file available from ECHA reports a Derived No Effect Level (DNEL) on the oral route for the general population of

0.85 mg/kg bw/d based on read-across to DEHA, where a NOAEL of 170 mg/kg bw/d was identified in a one-generation study on the basis of reduced body weight gain in maternal animals and offspring (ECHA, 2020d).

Exposure of the general population to DINA is likely, considering its consumer applications, including food contact materials (outside the EU), toys, and child care articles. Migration of DINA from PVC cling film into wrapped food has already been described (Saito et al., 2002; Tsumura et al., 2003; Kawamura et al., 2017; Carlos et al., 2018). Based on the DINA content of such cling films and the average food consumption, a daily intake of 21 µg/kg bw/d was estimated for the Japanese population from that exposure source (Kawamura et al., 2017). In Germany, however, DINA has been detected only in 5% of house dust samples from daycare centers with a maximum concentration of 34 mg/kg, and thus at much lower incidence rates and concentrations than other plasticizers such as DEHP (Fromme et al., 2016). Human Biomonitoring (HBM) provides an integral measure for exposure assessment covering all routes of uptake (oral, inhalation, dermal) and exposure sources and thus can be used for a robust exposure and risk assessments (Needham et al., 2007; Angerer et al., 2007; Schindler et al., 2014; Schwedler et al., 2017; Kolossa-Gehring et al., 2017; Haines et al., 2017). We have already presented such an approaches for the adipate di(2-ethylhexyl) adipate (DEHA) (Nehring et al., 2020) and similar approaches have been established by us and others for a wide range of phthalate plasticizers and their alternatives such as di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH) and di(2-ethylhexyl) terephthalate (DEHTP) (Zota et al., 2014; Silva et al., 2017; Koch et al., 2017; Schwedler et al., 2020a, 2020b, 2020c; Lessmann et al., 2019; Kasper-Sonnenberg et al., 2019; Frederiksen et al., 2020). Thus, the aim of this study was to identify human metabolites of DINA in urine as specific DINA exposure biomarkers, to investigate their elimination kinetics in order to obtain FUEs for an objective DINA exposure and risk assessment, and to apply this approach in a small pilot population.

6.2. Material and methods

6.2.1. Chemicals

Diisononyl adipate (≥99.5%) used in the oral dosing study was provided by BASF SE (Ludwigshafen, Germany). The analytical standards of the DINA metabolites 1-mono-(4-methyl-7-hydroxyoctyl) adipate (7OH-MINA; >97%), 1-mono-(4-methyl-7-oxooctyl) adipate (7oxo-MINA; >97%), and 1-mono-(4-methyl-7-carboxyheptyl) adipate (7cx-MIOA; >97%), as well as DEHA metabolites (for simultaneous determination of DINA and DEHA metabolites; see Supplementary material) 1-mono-(2-ethyl-5-hydroxyhexyl) adipate (5OH-MEHA; >95%), 1-mono-(2-ethyl-5-oxohexyl) adipate (5oxo-MEHA; >97%), 1-mono-(2-ethyl-5-carboxypentyl) adipate (5cx-MEPA; >97%), and their respective 13C6-labeled analogs (13C6-7OH-MINA, 13C6-7cx-MIOA, 13C6-5OH-MEHA, 13C6-5oxo-MEHA, and 13C6-5cx-MEPA; same chemical purity as corresponding unlabeled chemicals, (labels in the adipic acid moiety) were custom synthesized by Dr. Vladimir Belov (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). Identities and purities of all labeled and non-labeled standard substances were confirmed by ESI-MS and 1H NMR. Water, acetonitrile and methanol (all CHROMASOLVTM LC-MS), ethanol (≥99.8%), and formic acid (puriss p.a.) were purchased from Honeywell (Seelze, Germany). Acetic acid (100% for LC-MS) was purchased from Merck (Darmstadt, Germany). Ammonium acetate BioXtra (≥98%) was purchased from Sigma Aldrich (Steinheim, Germany). Pure β-glucuronidase (without arylsulfatase/esterase activity) from E. coli K12 was purchased from Roche Diagnostics (Mannheim, Germany).

6.2.2. Dosing study

Three healthy German volunteers (2 females, 1 male; aged between 25 and 37 years; bodyweight between 67 and 86 kg), all without known occupational exposure to DINA, received a single oral dose of approximately 10 mg DINA (weighed precisely), resulting in individual doses between 113 to145 µg/kg bw, being roughly a factor of 6-8 below the long-term DNEL of DINA of 850 µg/kg bw/d. The DINA dose was dissolved in 1 mL ethanol and diluted with 5 mL water and was provided in a chocolate coated waffle cup. After receiving the DINA dose, the volunteers had a small breakfast.

Full void urine sample were collected immediately before the dose (t=0), as well as for 48 h after dose in separate 250 mL polyethylene containers and stored frozen at -20 °C until further use. Sampling times were noted by the volunteers and sample volumes were determined by the mass difference between empty and filled sample containers. Urinary creatinine concentrations were determined by L.u.P GmbH Labor- und Praxisservice (Bochum, Germany).

6.2.3. Pilot population

In addition to the metabolism study, convenience spot urine samples from a small pilot population were analyzed for DINA metabolites. The pilot population consisted of 35 German volunteers (age 23 to 59 (median 42), 24 females and 11 males), not occupationally exposed to DINA. Urine samples had been collected in April 2017.

6.2.4. Chemical analyses of DINA (and DEHA) metabolites

The secondary oxidized DINA metabolites mono-(hydroxyisononyl) adipate (OH-MINA), mono-(oxoisononyl) adipate (oxo-MINA), and mono-(carboxyisooctyl) adipate (cx-MIOA) were analyzed using a newly developed online-SPE-LC-MS/MS method, based on a method for DEHA metabolites, previously published by our group (Nehring et al., 2019). The DEHA metabolites 5OH-MEHA, 5oxo-MEHA, and 5cx-MEPA were also included in the method because of shared fragmentation patterns and potential chromatographic overlaps. In brief, 300 µL urine (or calibration solution) were mixed with 100 µL ammonium acetate buffer (1 M, pH = 6.0 – 6.4), 20 µL internal standard solution ($^{13}C_6$-labeled standards in water), and 6 µL β-glucuronidase (premixed 1:1 with ammonium acetate buffer). After enzymatic deconjugation of glucuronic acid conjugates (2 h, 37°C), 30 µL formic acid were added and the samples were frozen over night at -20°C to precipitate cryophobic proteins. Samples were thawed at room temperature, centrifuged (10 min at 1900 g) and 25 µL of the supernatant were analyzed by liquid chromatography (LC) , using a phenylhexyl-modified silica gel column with superficially porous particles (Kinetex® Phenyl-Hexyl 150x3 mm, particle size 2.6 µm; with SecurityGuard™ ULTRA Cartridges UHPLC Phenyl 3.0mm ID Columns; Phemomenex, Aschaffenburg, Germany) for chromatographic separation, coupled with online turbulent flow chromatography for matrix depletion and analyte enrichment (TurboFlow® Phenyl 50 x 0.5 mm; Thermo ScientificTM, Franklin, MA, USA) (online-

SPE). Detection was performed by electrospray ionization-triple quadrupole-tandem mass spectrometry (ESI-MS/MS) in negative ion mode using the time-programmed multiple reaction monitoring (scheduled MRM) detection mode. For a more detailed description see supplementary material.

6.2.5. Statistics

Data analysis was conducted with Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Urinary excretion fractions (F_{UE}s) were calculated, using the following equation (∑mi: sum of masses of the respective metabolite in all urine samples, M(DINA): molar mass of DINA, M(Metabolite): molar mass of the respective metabolite, and D the absolute applied DINA dose):

$$F_{UE} = \frac{\sum m_i * \left(\frac{M(DINA)}{M(Metabolite)}\right)}{D} * 100\%$$

6.3. Results and discussion

6.3.1. DINA metabolites under investigation and their quantification

The metabolism pathways leading to the postulated oxidized monoester metabolites OH-MINA, oxo-MINA and cx-MIOA, and adipic acid (AA) after full hydrolyses of DINA are shown in **Fig. 1** (for simplification, only the 4-methyloctyl based isomers are shown for each metabolite). Based on the knowledge on human DEHA and DnBA (publication in preparation) metabolism, AA will most likely be the ultimate product of hydrolytic DINA metabolism and a major metabolite of DINA. However, AA is not specific for DINA exposure (not even as a sum parameter for adipic acid esters because AA itself is a registered food additive (E 355) and food contact material in Europe (FCM 303)) and high background concentrations were observed in the general population (Nehring et al., 2020; for DnBA publication in preparation). Thus AA was not investigated in this study.

Considering the complexity of the isomeric composition of DINA itself, as well as a variety of possible positions for oxidative functionalization by phase I metabolism, it is not feasible to cover all isomers with individual analytical standards. Instead, we obtained defined analytical standards based on the 4-methyloctyl isomer, which has been shown as a major isomer in industrial isononyl alcohol (INA) (Koch et al., 2007) with oxidative modifications at position 7 (OH- and oxo-MINA) or 8 (cx-MIOA) of the alkyl

side-chain. For OH-MINA and cx-MIOA, which were expected to be of greater quantitative relevance compared to oxo-MINA based on experiences with DEHA and DnBA (Nehring et al., 2020; for DnBA publication in preparation), we also obtained $^{13}C_6$-labeled internal standards.

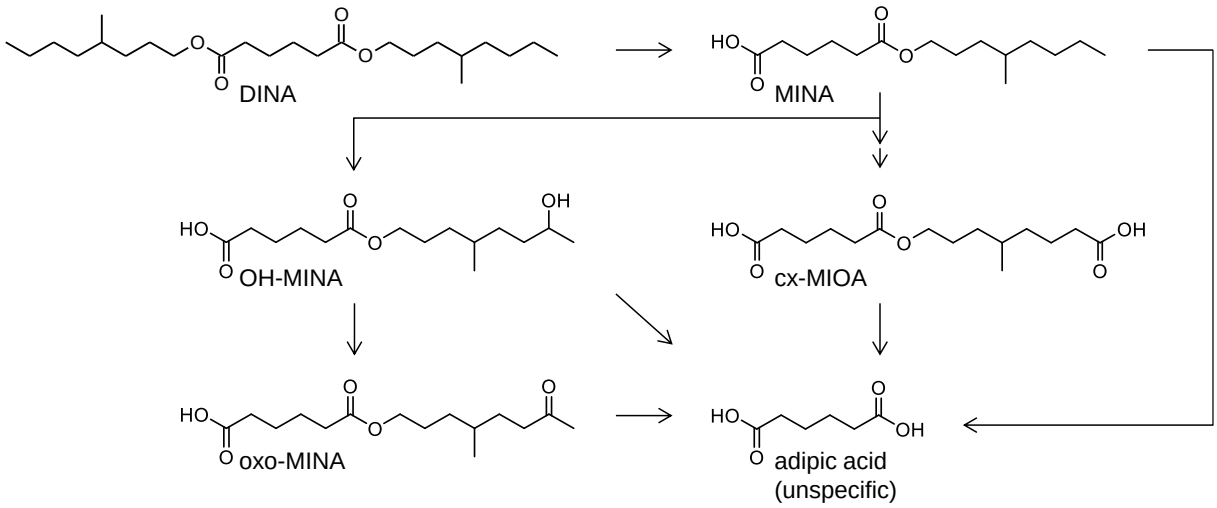


Fig. 1: *Human metabolism pathways of DINA. The side chain oxidized monoester metabolites OH-MINA, oxo-MINA and cx-MIOA (only isomers based on the 4-methyloctyl sidechain shown for simplification) were confirmed and quantitatively investigated in the current study. Furthermore, in analogy to human DEHA and DnBA metabolism, adipic acid (AA) is assumed as the ultimate breakdown product (not investigated in this study). For simplification, phase two metabolites (e.g. glucuronic acid conjugates) are not shown*

Fig. 2 shows exemplary chromatograms of the three target metabolites in a calibration standard in water (left column), a pre-dose, general population urine sample (middle column), and a post-dose urine sample (right column). Narrow single chromatographic peaks were obtained for each analytical standard (labeled and unlabeled) due to the single oxidized 4-methyloctyl isomer that was used as a representative of all possible DINA metabolites. No DINA metabolites could be detected in the pre-dose urine sample. The two peaks observed in the cx-MIOA traces did not belong to DINA because these peaks did neither increase after the DINA dose nor follow any other observable kinetic. In urine samples after the oral dose, characteristic peak patterns emerged (right column), eluting within a time-frame of roughly 1.5 minutes, reflecting the complex mixture of DINA metabolite isomers. As can be seen, the 4-methyloctyl isomer standards elute in the midst of the isomeric peak pattern, but do not necessarily represent the major peak. Quantification was performed by cumulative integration of all isomers of each metabolite (i.e., OH-MINA, oxo-MINA, and cx-MIOA) and via the calibration curve obtained for the respective monoisomeric analytical standard. Although some of the isomers might not be ideally quantified this way, this type of consensus method approach has already been established for other

isomeric plasticizers with isononyl alkyl chains such as diisononyl phthalate DINP (Wittassek et al., 2007; Kochand Angerer, 2007; Koch et al., 2007; Anderson et al., 2011; Koch et al., 2017; Frederiksen et al., 2020; Apel et al., 2020) or DINCH (Silva et al., 2013; Koch et al., 2013; Schütze et al., 2014; Kasper-Sonnenberg et al., 2019). Within the isomeric peak patterns, we observed different quantifier/qualifier ratios (up to 2-fold difference) and consequently also somewhat different quantifier/qualifier ratios for the analytical standards on one hand and the respective native metabolites (sum of peak areas of all isomers) on the other hand. This indicated that the exact constitution of the different isononyl isomers, as well as the position of the functional groups has an influence on the fragmentation behavior of the metabolites. However, quantifier/qualifier ratios for the urine samples were within narrow limits for each individual metabolite (again, sum of isomers), despite the differences observed in comparison to the monoisomeric standards.

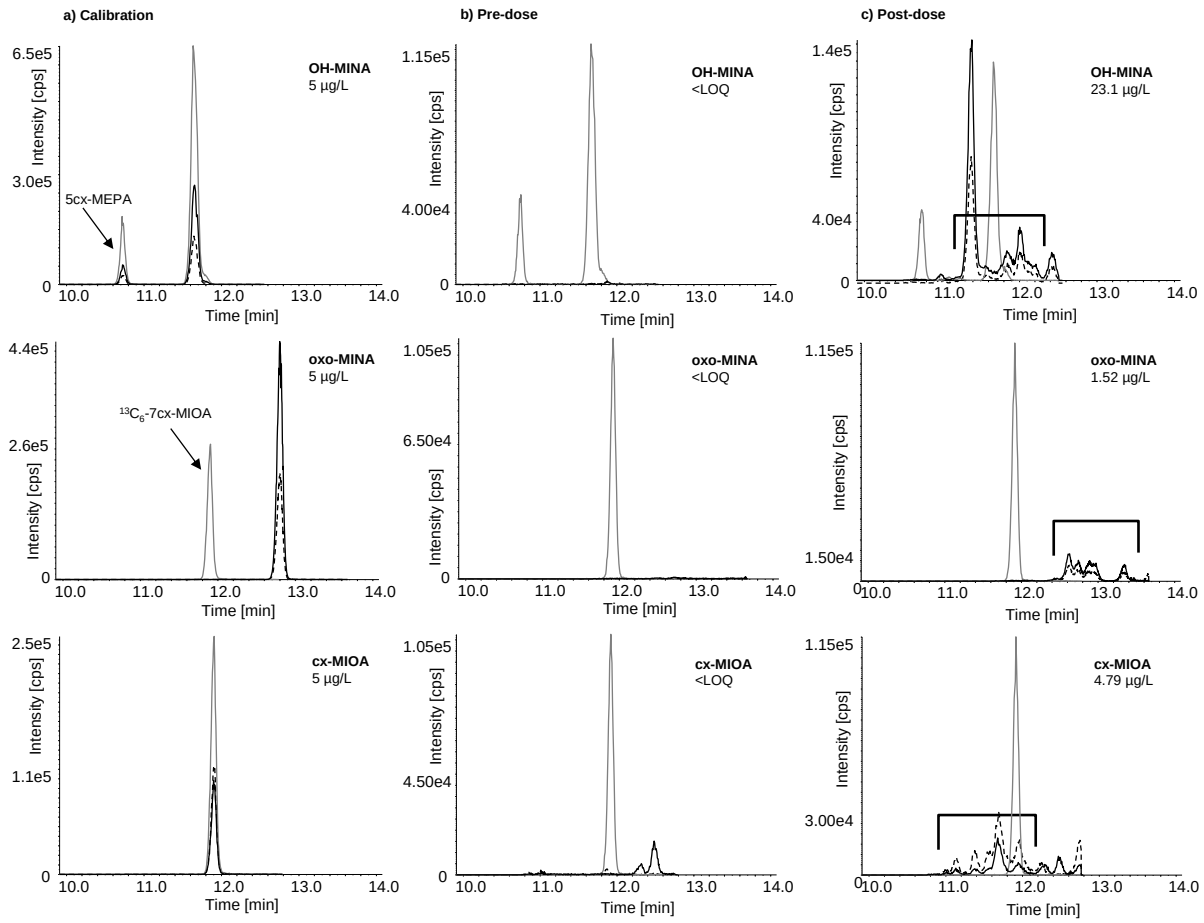


Fig. 2: *Chromatograms of (a) a calibration standard in water, (b) a urine sample collected before dose (OH-MINA, oxo-MINA, and cx-MIOA below the LOQ; only the internal standard peaks can be seen), and (c) a urine sample collected 2 h after oral dose. Native DINA metabolite traces shown in black (quantifier transitions as continuous lines and qualifier transitions in dotted lines) and labeled internal standard traces in gray. The time frame of elution of the dose related metabolite isomers in (c) is indicated by open brackets*

Another aspect to be pointed out is that the DINA metabolite OH-MINA and the DEHA metabolite 5cx-MEPA are two isobaric compounds (or mixture of compounds in case of OH-MINA) and OH-MINA shares all its (adipic-acid derived) mass fragments with 5cx-MEPA. Specific sidechain-derived fragments were only detected for 5cx-MEPA but not for OH-MINA. Therefore, special attention had to be paid to the chromatographic separation of 5cx-MEPA from the OH-MINA isomers, which could be achieved as can be seen in the post dose chromatogram (right column, upper row) with 5cx-MEPA eluting before the OH-MINA isomeric peak pattern.

The accuracy, determined through spiking of three different concentration levels of the authentic standard substances to eight different urine samples (creatinine content 0.34 to 2.3 g/L) was very satisfactory (relative recoveries 90-121%) for all DINA and DEHA monoester metabolites. For oxo-MINA, with no authentic internal standard available ($^{13}C_6$-7cx-MIOA was used as a surrogate internal standard) we determined relative recoveries of 38-112%, indicating to problems in some urine samples. Excluding one urine sample with recovery issues, relative recoveries in the other seven samples were in a satisfactory range (72-112%). Nevertheless, we currently regard oxo-MINA results as semi-quantitative. The method's precision was satisfactory for all metabolites with intra- and interday coefficients of variation ≤12% (≤7.6%, excluding oxo-MINA). The limits of quantification (LOQ) (based on a signal-to-noise ratio of 10 in native urine samples containing the target analytes) were 0.3 µg/L (OH-MINA and oxo-MINA), 0.6 µg/L (cx-MIOA), 0.5 µg/L (5OH-MEHA), 0.1 µg/L (5oxo-MEHA), and 0.05 µg/L (5cx-MEPA). For more details, see Supplementary material.

6.3.2. Elimination kinetics of DINA monoester metabolites after oral dose

After the single oral DINA dose, the three volunteers donated 24, 23, and 29 individual, full volume urine samples with a total urine volume of 3824, 2044, and 3020 mL over the period of 48 hours. In none of the pre-dose samples, any of the three oxidized DINA metabolites could be detected. However, they quickly emerged in the post dose samples (see **Fig. 2**, right column, for exemplary chromatograms) with maximum urinary concentrations (c_{max} see **table 1**, sum of isomers) at least a factor of 55 (OH-MINA), 5 (oxo-MINA), and 8 (cx-MINA) above the respective LOQs.

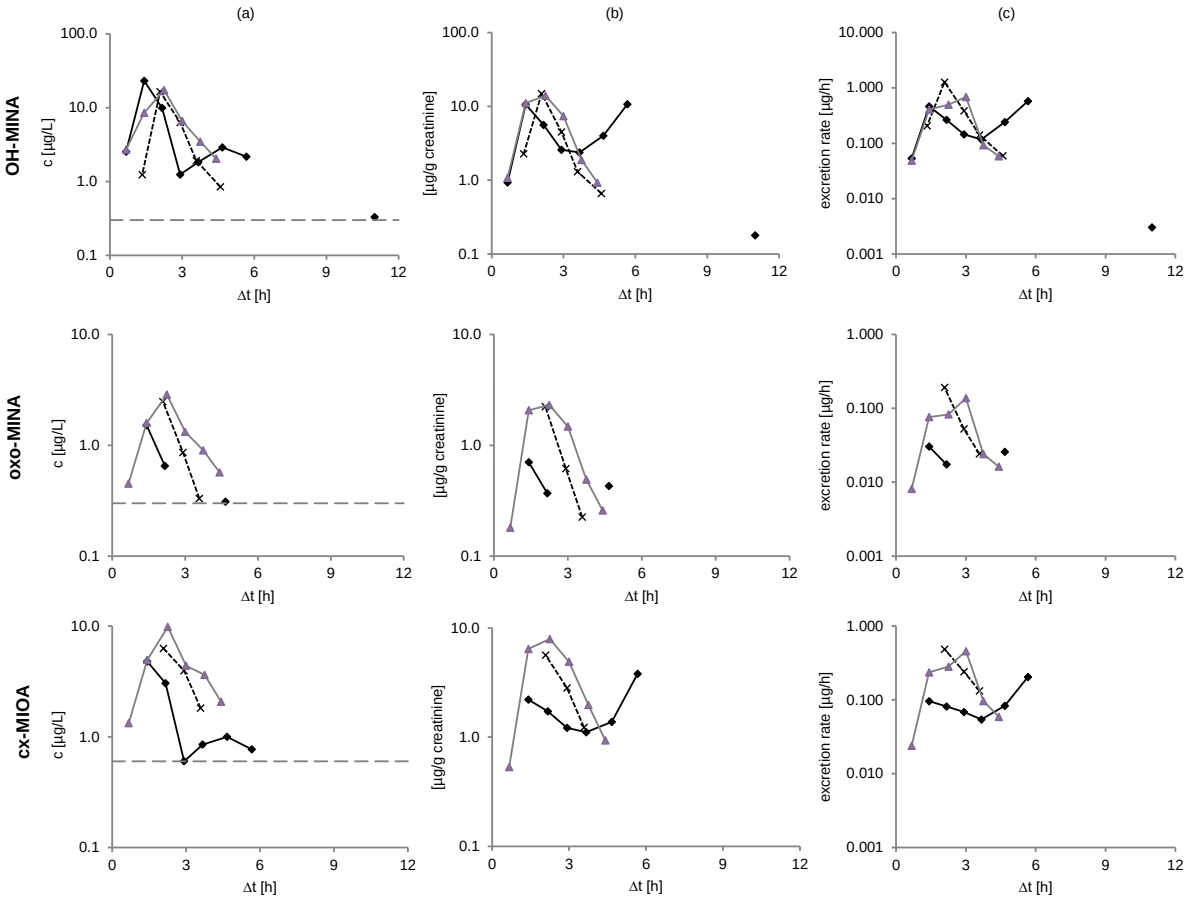


Fig. 3: *Elimination kinetics of OH-MINA (top), oxo-MINA (middle), and cx-MIOA (bottom) for all three volunteers after oral DINA dose. Unadjusted concentrations in µg/L with respective LOQs shown with gray dotted lines are presented in column (a), creatinine-adjusted concentrations in µg/g creatinine in column (b), and excretion rates in µg/h in column (c)*

Fig. 3 shows the urinary excretion kinetics for the three DINA monoester metabolites in all three volunteers. Absolute concentrations (in µg/L, left panel), creatinine-adjusted concentrations (in µg/g creatinine, middle), and the excretion rates (in µg/h, right panel) are shown on a logarithmic scale. For all three metabolites, urinary peak concentrations (c_{max} - see **table 1**) were observed between 1.4 and 2.3 h after oral dose. For one volunteer (depicted with black solid line), a second peak was observed at 5.7 h for OH-MINA and cx-MIOA (oxo-MINA was below the LOQ for this time point).

Table 1: *Elimination kinetics for OH-MINA, oxo-MINA, and cx-MIOA in three volunteers after a single oral dose. Peak concentrations (c_{max} – first maximum) after oral dose and their respective time points (t_{max}) (mean values; ranges in parentheses). Given the limited number of data points, elimination half-lives were not calculated*

	OH-MINA	oxo-MINA	cx-MIOA
t_{max} [h]	1.9*	1.9	1.9*
	(1.4-2.3)	(1.4-2.3)	(1.4-2.3)
c_{max} [µg/L]	19.0	2.29	6.97
	(16.5-23.1)	(1.52-2.87)	(4.79-9.83)
c_{max} [µg/g creatinine]	13.2*	1.75	5.27*
	(10.7-14.9)	(0.70-2.31)	(2.21-7.93)

* for one volunteer, a second concentration maximum was observed for OH-MINA (5.7 h, 10.7 µg/g creatinine) and cx-MIOA (5.7 h, 3.81 µg/g creatinine)

After the maximum (or maxima, in case of one volunteer), the metabolite concentrations rapidly fell below the respective LOQs in all volunteers after 4.7-5.7 h for OH-MINA (with one single sample from one volunteer above the LOQ at 11 h post dose), 2.2-4.4 h for oxo-MINA (with only one sample above the LOQ at 4.7 h post dose) , and 3.6-5.7 h for cx-MIOA. F_{UE}s calculated for these three side-chain oxidized DINA metabolites based on their total amounts excreted via urine are shown in **table 2.** In line with the peak concentrations observed **(table 1)**, OH-MINA was the major of the three DINA metabolites, accounting for 0.022% (mean) of the DINA dose. The F_{UE}s were very consistent between the three volunteers (0.020-0.023%). The two other metabolites were excreted at considerably lower dose shares (0.009% for cx-MIOA and 0.003% for oxo-MINA).

Table 2: *Urinary excretion fractions (F_{UE}s) of the DINA metabolites OH-MINA, oxo-MINA, and cx-MIOA after a single oral dose in three healthy volunteers (mean values; ranges in parentheses)*

	OH-MINA	oxo-MINA	cx-MIOA
0-6 h [%]			
Mean	0.022	0.003	0.009
(Range)	(0.020-0.023)	(0.001-0.004)	(0.007-0.012)
6- 24 h [%]			
Mean	0.000	0.000	0.000
(Range)	(0.000-0.000)	(0.000-0.000)	(0.000-0.000)
Total 0-24h [%]			
Mean	**0.022**	**0.003**	**0.009**
(Range)	**(0.020-0.023)**	**(0.001-0.004)**	**(0.007-0.012)**

In addition to the excretion kinetics of the specific DINA metabolites we investigated the glucuronidation patterns. To determine the total amounts (sum of glucuronidated and non-conjugated metabolite) of DINA metabolites pooled urine samples (0-6 h) from each volunteer were treated the same way as described in section “Chemical analyses of DINA (and DEHA) metabolites”. For the determination of free non-conjugated metabolites the same procedure without addition of β-glucuronidase was applied. The percentages of non-conjugated (‘free’) metabolites in relation to the total amounts was negligible (0-7%) for all three DINA metabolites and volunteers. Due to the strongly non-polar alkyl side-chain conjugation seems necessary to provide needed hydrophilicity for excretion via urine.

6.3.3. Comparison with other adipate esters

In total, only 0.034% of the oral DINA dose could be recovered in urine as the above three oxidized DINA monoester metabolites. This percentage is disappointingly low from the perspective of identifying sensitive and specific urinary exposure biomarkers for DINA. However, it is in line with urinary excretion characteristics known for other adipates.

For DEHA, the homologous adipate with shorter alkyl chains by one carbon, the oxidized monoester metabolites were excreted with a sum-F_{UE} of 0.32% (Nehring et al., 2020). For DnBA, the oxidized metabolites represented 0.49% (publication in preparation). Thus, these low F_{UE}s seem to further diminish with increasing alkyl chain lengths. Because we could show for DnBA and DEHA that the simple monoester was not excreted in relevant amounts via urine (Nehring et al., 2020; for DnBA publication in

preparation), the formation and elimination of mono-isononyl adipate (MINA) was not further investigated in this study. For DnBA and DEHA, metabolism to the hydrolysis product adipic acid has been shown to be of major importance (DEHA: 10-40%, DnBA: 14-26%) (Nehring et al., 2020; for DnBA publication in preparation). Further extensive metabolism resulting in carbon dioxide has been described for DEHA in rats (Takahashi et al., 1981). Therefore, we assume that rapid hydrolysis and elimination via non-specific adipic acid and further break-down to carbon dioxide will be of similar importance also for DINA. Because of the difficulties in adipic acid determination (due to high, fluctuating background levels already knoen for DEHA and DnBA) we waived the determination of adipic acid in the DINA metabolism samples.

6.3.4. DINA exposure assessment in a pilot population

The newly identified, specific DINA metabolites were analyzed in a pilot population from Germany (35 spot urine samples from the general population; all without known DINA exposure). Concentrations of all three DINA metabolites were below the respective LOQ. Nevertheless, the derived F_{UE}s enable the calculation of daily intakes (DI) in a worst case scenario, using the LOQ as upper bound metabolite concentrations. We performed the calculation of DIs according to Kohn et al., 2000 and Koch et al., 2003 as described in Nehring et al., 2020 on the example of DEHA. Based on the F_{UE} of the major metabolite OH-MINA worst case DIs for DINA were calculated to be lower than 50 µg/kg bw/d, ranging between 34 and 43 µg/(kg bw/d). Taking into account the DNEL of 850 µg/kg bw/day for DINA (ECHA, 2020d), this would indicate that worst case exposures of the pilot population would be at least a factor of 20 below the DNEL (oral route) for the general population. These low (non-detectable) DINA exposures in our (adult) German pilot population are in line with the main sources of DINA exposure expected from toys and childcare articles, as well as from migration from FCM into food (in Europe DINA is not permitted for use in FCM).

6.4. Conclusion

For the first time, data on human DINA metabolism and urinary excretion were provided. Three monoester metabolites with oxidative modifications (hydroxy, oxo, and carboxylic acid groups) in the isononyl (technical mixture, thus containing several isomers) sidechain were identified (OH-MINA, oxo-MINA, and cx-MIOA) as urinary metabolites of DINA. An analytical method for their determination was developed, capturing the sum of isomers of each of the metabolites. The urinary excretion of the oxidized DINA monoester metabolites was quantitatively investigated after oral dose in three volunteers. All three metabolites emerged quickly, reaching peak concentrations at around 2 h post dose. Based on their urinary excretion, urinary excretion fractions (F_{UE}s) were derived, which were low (sum of F_{UE}s ≤0.034%), but in a comparable range as those of the two adipates DEHA and DnBA. In analogy to these adipate plasticizers, adipic acid can be expected as a major metabolite of DINA (not investigated). Yet, its lack of specificity in conjunction with high background concentrations renders it useless as exposure biomarker. OH-MINA was the major specific DINA metabolite with inter-individually highly consistent F_{UE}s (0.020-0.023). In a pilot population of German adults, DINA monoester metabolite concentrations were below the LOQ. We expect higher DINA exposure in populations from countries which permit the use of DINA in FCM, but also in children due to DINA containing toys and childcare articles; or in occupationally exposed populations. For these populations the above presented DINA metabolites can be used as valuable and specific exposure biomarkers with our methods LOQ sufficiently low enough to be able to sensitively detect potential DNEL exceedances. The newly derived F_{UE}s in conjunction with the sensitive analytical HBM method allow to reliably assess DINA exposures and perform risk assessments based on daily intakes.

6.5. Acknowledgement

The development of the analytical method and its application in investigating human metabolism and population samples are part of a large-scale 10-year project on the advancement of human biomonitoring in Germany. This project is a cooperation agreed in 2010 between the Federal Ministry for the Environment, Nature Conservation, and Nuclear Safety (BMU) and the Verband der Chemischen Industrie e.V. (German Chemical Industry Association– VCI) and is managed by the German Environment Agency (UBA). Experts from governmental scientific authorities, industry and science accompany the project in substance selection and method development (Kolossa-Gehring et al., 2017). The analytical method development was financed by the Chemie Wirtschaftsförderungsgesellschaft mbH.

6.6. Compliance with ethical standards

6.6.1. Ethical Approval

The study has been reviewed by the Ethics-Commission of the medical faculty of the Ruhr University Bochum, Germany (IRB Reg. No.: 15-5422 and 3867-10). The study was performed in accordance with the Code of Ethics of the World Medical Association (1964 Declaration of Helsinki and its later amendments). The study design was presented to the participants in written form and written informed consent was obtained from each participant.

6.6.2 Conflict of interest

The participation of Rainer Otter as co-author was conducted as part of his employment responsibilities with BASF SE, a manufacturer of DINA, and his advisory role within the BMU-VCI cooperation project on human biomonitoring. The interpretation and views expressed in this manuscript are not necessarily those of the co-author´s employer.

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Supplementary Material

This supplementary material includes:

Further information on the chemical analyses of the specific DINA (and DEHA) metabolites.

S6.1. Chemical analysis:

S6.1.1. Chromatographic conditions:

A 1260 Infinity HPLC system (Agilent Technologies, Waldbronn, Germany) was used, consisting of a G1367E autosampler, a G1311B quaternary pump, a G1312B binary pump coupled with a G4225A vacuum degasser, and a G1316A thermostated column compartment with a 6-port valve. The quaternary pump was connected with the autosampler and was used as loading pump, while the binary pump was connected with the analytical column (via the 6-port valve) for chromatographic separation. The 6-port valve was used to direct the eluent flow from both pumps to operate the two-column assembly. In position A ("loading position") the flow from the quaternary pump was used to transfer the sample from the autosampler, via the 6-port valve, onto the enrichment column and from there (again via 6-port valve) into the waste. Simultaneously, the flow from the binary pump was directed (via the 6-port valve) onto the analytical column (which was coupled to the MS). For transfer of the analytes from the enrichment column to the analytical column, the valve was switched to position B ("transfer position"). In that position, the flow from the binary pump was directed onto the enrichment column and then onto the analytical column, while the flow from the quaternary pump was diverted directly into the waste. After analyte transfer was complete, the valve was switched back to position A for the chromatographic analysis.

As eluents water (solvent A) and acetonitrile (solvent B), each containing 0.05% acetic acid, were used for both pumps. The solvent gradients used for analyte enrichment and for chromatographic separation are shown in **table S1** and **table S2**. The flow rate on the binary pump was kept at 300 µL/min.

Table S1: *Solvent gradient for analyte enrichment (quaternary pump)*

Time [min]	Flow [µL/min]	Eluent A [%]	Eluent B [%]
0	1500	100	0
3.5	1500	100	0
4	500	100	0
6	500	100	0
12	500	5	95
22	500	5	95
23	500	100	0
24	1000	100	0
25	1500	100	0
27	1500	100	0

Table S2: *Solvent gradient for the chromatographic separation (binary pump)*

Time [min]	Eluent A [%]	Eluent B [%]
0	75	25
2.5	75	25
3.5	65	35
9	55	45
14	5	95
23	5	95
23.5	75	25
27	75	25

The gradient delay volumes according to the manufacturer´s specifications were 600 - 800 µL for the binary pump flow path and 870 - 1170 µL for the quaternary pump flow path (including 270 µL gradient delay volume of the autosampler). For analyte enrichment and matrix depletion the switching valve was kept in position A for three minutes. Afterwards, the 6-port valve was switched to position B for analyte transfer and switched back after 2 min (5 min of total runtime) for chromatographic separation and flushing/re-equilibration of the enrichment column.. The column compartment was kept at 25±1 °C. The injection process includes a needle wash with methanol/water 8:2 (v/v) for 10 s.

S6.2.2. Mass spectrometric conditions:

An AB Sciex 4500 triple quadrupole mass spectrometer (Darmstadt, Germany) was used for detection. The instrument voltages were -4.5 kV (ion spray voltage), -10 V (entrance potential), and -11 V (collision cell exit potential). Instrument gases (nitrogen) were set to 35 psi (curtain gas), 55 psi (nebulizer gas), 50 psi (heater gas), and 6 arbitrary units (collision gas). The source heater temperature was set to 550 °C. DINA and DEHA metabolites were detected in two separate MRM experiments with detection windows of 120 s (DINA metabolites) and 40 s (DEHA metabolites) and target scan times of 0.3 s. Further scheduled MRM conditions (collision energies (CE) and declustering potentials (DP)) were optimized manually for each standard substance (**table S3)**. In case of DINA metabolites, conditions determined for the single isomeric standard substances were applied to the totality of metabolite isomers present in native urine samples. Analyst 1.6.2 was used for instrument control and MultiQuant 3.0.2 for quantitative data analysis (both Sciex, Darmstadt, Germany).

Table S3: *Time programmed MRM parameters for each analyte*

	t_R [min]	*m/z* precursor ion	*m/z* product ion	DP [V]	CE [eV]
OH-MINA					
quantifier	11.50	287	83	-80	-25
qualifier			127		-20
$^{13}C_6$-OH-MINA					
quantifier	11.50	293	88	-80	-25
qualifier			133		-20
oxo-MINA					
quantifier	12.66	285	83	-80	-20
qualifier			127		-15
cx-MIOA					
quantifier	11.76	301	83	-80	-30
qualifier			145		-23
$^{13}C_6$-cx-MIOA					
quantifier	11.76	307	88	-80	-30
qualifier			173		-25
5OH-MEHA					
quantifier	10.31	273	83	-80	-23
qualifier			127		-16
$^{13}C_6$-5OH-MEHA					
quantifier	10.31	279	86	-80	-45
qualifier			133		-18
5oxo-MEHA					
quantifier	11.45	271	83	-80	-20
qualifier			127		-15
$^{13}C_6$-5oxo-MEHA					
quantifier	11.45	277	88	-80	-21
qualifier			133		-16
5cx-MEPA					
quantifier	10.63	287	145	-80	-22
qualifier			101		-35
$^{13}C_6$-5cx-MEPA					
quantifier	10.63	293	151	-75	-21
qualifier			88		-35

MRM: multiple reaction monitoring t_R: retention time, DP: declustering potential, CE: collision energy

Fig. S1 shows the product ion spectra with postulated fragment structures of the unlabeled single isomer DINA metabolite standards (4-methyloctyladipate with oxidative modifications in position 7 or 8 of the alkyl side-chain). Fragments attributable to adipic acid were observed at *m/z* 81, 83, 101, and 127 and have already been described for DEHA (Nehring et al., 2019). A fragment at *m/z* 145 (adipic acid) was only observed for 7cx-MINA. This is in line with observations made for DEHA and DnBA metabolites (Nehring et al., 2019;publication in preparation in case of DnBA). For 7cx-MIOA, specific alkyl side-chain fragments were observed at *m/z* 173 and *m/z* 127, corresponding to *m/z* 159 and *m/z* 113 for 5cx-MEPA (Nehring et al. 2019). Additionally, the 7cx-MIOA molecule can eliminate water resulting in *m/z* 283, followed by elimination of CO_2 to yield *m/z* 239. All postulated fragments were verified by their accurate masses using Q-Orbitrap-MS (below ±5 ppm; Q Exactive Focus, Thermo Scientific, Bremen, Germany) in ESI negative mode (ion spray voltage 2.5 kV, heater temperature 412 °C, ion transfer capillary temperature 256 °C) at maximum resolution setting (R = 70,000). The instrument gases (nitrogen) were set as follows: sheath gas 47.5 arbitrary units, auxiliary gas 11.25 arbitrary units. The S-Lens RF Level was set to 50 (data not shown). Corresponding $^{13}C_6$-labeled internal standards lead to the same fragments, only with the respectively higher masses (**Fig. S1)**.

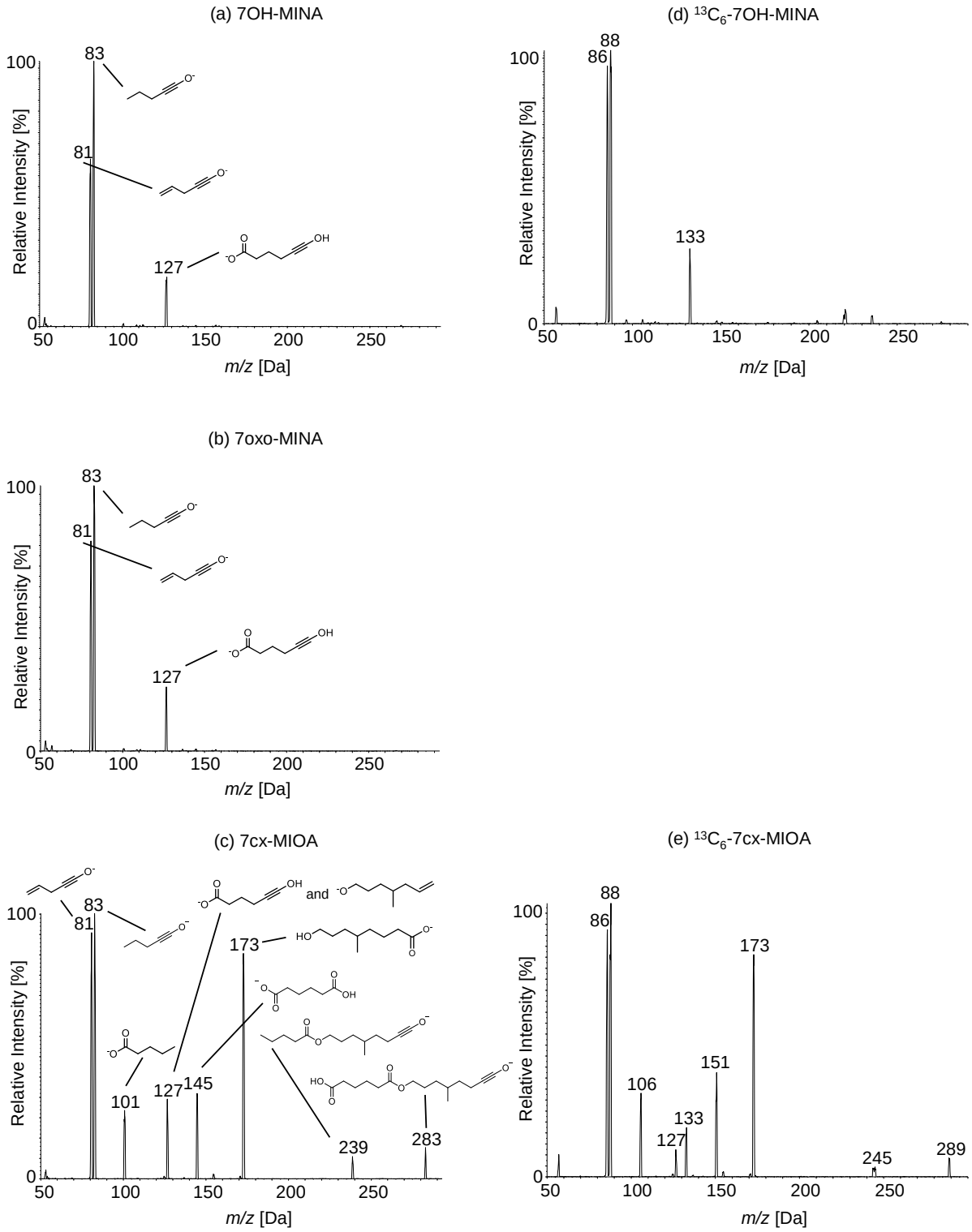

Fig. S1: *Product ion spectra of the unlabeled single isomeric standard substances 7OH-MINA (a), 7oxo-MINA (b), 7cx-MIOA (c), $^{13}C_6$-7OH-MINA (d), and $^{13}C_6$-7cx-MINA (e) recorded via direct infusion of the individual standard solutions (1 mg/L dissolved in acetonitrile/water 50:50 (v/v), containing 0.05% acetic acid) at 7 µL/min*

S6.1.3. Calibration, validation, and quality control

A stock solution (1g/L) of each unlabeled DEHA and DINA metabolite standard (7OH-MINA, 7oxo-MEHA, 7cx-MIOA, 5OH-MEHA, 5oxo-MEHA, 5cx-MEPA) and their $^{13}C_6$-labeled analogs (except for 7oxo-MINA, for which an internal standard was not available - instead, $^{13}C_6$-7cx-MIOA was used as a surrogate internal standard.) was prepared in acetonitrile. An aqueous internal standard solution containing 150 µg/L of each ^{13}C-labeled standard was prepared from the respective stock solutions. Calibration solutions (containing the non-labeled standards) with concentrations ranging between 0.05 µg/L and 22 µg/L were prepared in water. All solutions were stored in glass flasks, capped with screw caps with silicone/teflon septa until further use. Calibration curves, obtained by weighted (concentration^{-1}) linear regression, were linear throughout the entire calibration range (0.05 µg/L and 22 µg/L) for all metabolites (r ≥0.999). The limit of quantification (LOQ) of each metabolite was estimated based on a signal-to-noise-ratio (S/N) of 10 in native urine samples containing the target analytes. For the multi-isomeric DINA metabolites, the LOQ was attributed to concentrations resulting in an S/N ratio of 10 for the major isomeric peak.

For the determination of method precision and for quality control, quality control material at three different concentration levels (Q_{low}, Q_{med}, and Q_{high}) was prepared by pooling individual spot urine samples containing native DINA and DEHA metabolite concentrations . This material was treated the same way as urine samples and was analyzed in each analytical batch. In the Q_{high} material, DEHA metabolite levels were obtained by spiking (using authentic standards). To determine the precision of the method, Q_{low}, Q_{med}, and Q_{high} were analyzed six times within one series (intra-day precision) as well as on six different days (inter-day precision). Imprecision of the method (both intra- and inter-day) was ≤12% for all analytes (**table S4**).

Table S4: *Precision data of the method. All quality control samples (Q_{low}, Q_{med}, Q_{high}) were prepared from urine samples containing native DINA and DEHA metabolite concentrations (except for Q_{high}, in which DEHA metabolites were spiked using analytical standards)*

Analyte	**Intraday (n=6)**						**Interday (n=6)**					
	Q_{low}		Q_{med}		Q_{high}		Q_{low}		Q_{med}		Q_{high}	
	Mean [µg/L] (range)	RSD [%]	Mean [µg/L] (range)	RSD [%]	Mean [µg/L] (range)	RSD [%]	Mean [µg/L] (range)	RSD [%]	Mean [µg/L] (range)	RSD [%]	Mean [µg/L] (range)	RSD [%]
OH-MINA	0.76 (0.73-0.79)	2.9	3.35 (3.30-3.41)	1.2	35 (34.5-35.4)	0.8	0.7 (0.64-0.76)	5.8	3.37 (3.23-3.64)	4.2	32.9 (29.6-35.0)	5.5
oxo-MINA	<LOQ (<LOQ)	N/A	0.51 (0.49-0.52)	1.9	2.24 (2.20-2.29)	1.6	<LOQ (<LOQ)	N/A	0.51 (0.50-0.55)	3.6	1.94 (1.64-2.24)	12
cx-MIOA	<LOQ (<LOQ)	N/A	1.93 (1.79-2.12)	5.9	15.4 (14.9-15.8)	2.7	<LOQ (<LOQ)	N/A	1.84 (1.63-1.94)	6.4	14.4 (13.3-15.6)	7.5
5OH-MEHA	<LOQ (<LOQ)	N/A	0.54 (0.53-0.57)	3.8	7.38 (6.97-7.63)	3.4	<LOQ (<LOQ)	N/A	0.53 (0.48-0.58)	6.9	7.28 (6.98-7.58)	7.6
5oxo-MEHA	<LOQ (<LOQ)	N/A	0.47 (0.46-0.48)	1.6	7.01 (6.89-7.08)	1.1	<LOQ (<LOQ)	N/A	0.46 (0.44-0.49)	4.5	6.9 (6.70-7.15)	2.3
5cx-MEPA	0.48 (0.46-0.49)	2.8	8.07 (7.85-8.25)	2	13.9 (13.6-14.1)	1.5	0.46 (0.44-0.49)	4.1	7.81 (7.41-8.35)	4.7	13.4 (12.6-14.2)	4.3

RSD: relative standard deviation

Accuracy (in terms of relative recovery) and ruggedness in regard to differences in urinary matrix composition were determined by analyzing eight different urine samples with varying creatinine concentrations (0.3 – 2.3 g/L), both spiked at three different concentration levels (1, 5, and 15 µg/L), as well as without spiking. Relative recoveries were calculated after subtraction of native concentrations measured in the unspiked samples. For oxo-MINA, for which no authentic internal standard was available, in one urine sample relative recoveries around 40% were observed over all spiking levels, whereas relative recoveries in the remaining samples ranged between 72 and 112%. For all other metabolites, for which authentic internal standards were available, relative recoveries were between 90 and 121% (**table S5**). No relation between creatinine content and relative recoveries was observed for any of the analytes.

Table S5: *Accuracy (relative recovery) calculated from analysis of eight different urine samples with different creatinine concentrations (0.34 to .3 g/L creatinine) and spiked with approximately 1 µg/L, 5 µg/L and 15 µg/L*

Analyte	Native concentrations [µg/L]	Low			Medium			High		
		Spiked concentrations [µg/L]	Measured concentrations[a] [µg/L]	Accuracy [%]	Spiked concentrations [µg/L]	Measured concentrations[a] [µg/L]	Accuracy [%]	Spiked concentrations [µg/L]	Measured concentrations[a] [µg/L]	Accuracy [%]
OH-MINA	<LOQ	0.97	1.06 (0.94-1.12)	109 (97-115)	4.67	4.87 (4.52-5.19)	104 (97-111)	17.5	18.7 (17.0-19.5)	107 (97-111)
oxo-MINA	<LOQ	0.97	0.83 (0.44-1.09)	85 (45-112)	4.67	3.66 (1.78-4.73)	78 (38-101)	17.5	14.3 (7.53-18.2)	82 (43-104)
cx-MIOA	<LOQ	0.84	0.86 (0.78-0.92)	102 (93-110)	4.03	4.06 (3.79-4.24)	101 (94-105)	15.1	15.9 (14.8-16.7)	105 (98-110)
5OH-MEHA	<LOQ	0.89	0.93 (0.85-1.00)	105 (96-112)	4.27	4.17 (3.84-4.40)	98 (90-103)	16.0	16.5 (15.3-17.7)	103 (96-111)
5oxo-MEHA	<LOQ	0.86	0.91 (0.86-0.95)	105 (96-110)	4.14	4.29 (4.00-4.51)	104 (97-109)	15.5	16.8 (15.5-17.5)	110 (101-113)
5cx-MEPA	<LOQ-0.15	0.77	0.89 (0.82-0.93)	116 (107-121)	3.68	4.10 (3.92-4.34)	111 (106-118)	13.8	16.2 (14.7-16.6)	117 (106-120)

Mean values (ranges in parenthesis) for measured concentrations and accuracies; [a]background levels substracted; LOQ: limit of quantification (OH-MINA and oxo-MINA: 0.3 µg/L, cx-MIOA: 0.6 µg/L, 5OH-MEHA: 0.5 µg/L, 5oxo-MEHA: 0.1 µg/L, and 5cx-MEPA: 0.05 µg/L)

References

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Ringbeck B, Bury D, Hayen H, Weiss T, Brüning T, Koch HM. Determination of di-n-butyl adipate (DnBA) metabolites as possible biomarkers of exposure in human urine by online-SPE-LC-MS/MS. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci 2020;1141:122029.

Kapitel 7

Zusammenfassung und Ausblick

Das Ergebnis dieser Arbeit ist die erstmalige Entwicklung eines umfassenden Human-Biomonitoring-Ansatzes (inklusive Aufklärung des Humanmetabolismus und Ausscheidungskinetik) für den Weichmacher DEHA. Dadurch wurde die Wissenslücke zu einer validen Expositionsbewertung geschlossen und ein wichtiger Beitrag zur Risikoabschätzung der Allgemeinbevölkerung sowie zusätzlich exponierter Bevölkerungsgruppen geleistet.

Für die Erfassung der inneren Exposition wurde eine analytische Methode für die sensitive und quantitative Bestimmung von drei spezifischen DEHA-Metaboliten entwickelt. Wie bei Phthalaten und anderen Substituten, die ebenfalls eine 2-Ethylhexyl-Alkylkette enthalten, stellen auch bei DEHA spezifische Metaboliten mit oxidativen Modifikationen in der Seitenkette die bestmöglichen Expositionsbiomarker dar, da sie in ausreichender Menge ausgeschieden werden und aufgrund ihrer Spezifität kontaminationsunanfällig sind. Für die Erfassung der freien und mit Glucuronsäure konjugierten Metaboliten im menschlichen Urin wurden die Proben einer enzymatischen Hydrolyse unterworfen (quantitative Rückführung auf die jeweiligen Phase I Metaboliten) und anschließend mittels online-SPE-LC-MS/MS analysiert. Die Quantifizierung über SIVA mit authentischen $^{13}C_6$-markierten Internen Standards wurde erstmalig für alle drei Metaboliten durchgeführt. Die entwickelte Methode zeichnet sich durch einen hohen Automatisierungsgrad, ein hohes Maß an Sensitivität (Bestimmungsgrenze in Urin (LOQ, engl. *limit of quantification*) 5cx-MEPA und 5OH-MEHA: 0,05 µg/L, 5oxo-MEHA: 0,1 µg/L), Spezifität und Robustheit (relative Wiederfindung: 92-109%; relative Standardabweichung <5%) aus.

Wie im Rahmen der Entwicklung einer neuen HBM-Methode üblich, wurde die Eignung der analytischen Methode und der Expositions-Biomarker anhand von Urinproben erster Pilotpopulationen überprüft. Untersucht wurden 32 Urinproben aus Deutschland, 44 Urinproben schwangerer Frauen aus Brasilien sowie Urinproben von 6 Probanden, welche nach dem Verzehr von in DEHA-haltiger Frischhaltefolie verpackten Lebensmitteln gesammelt wurden. Im Urin dieser 6 Probanden konnten alle drei DEHA Expositionsbiomarker detektiert und quantifiziert werden, was die hinreichende Eignung dieser Biomarker im Hinblick auf erwartbare Expositions-Szenarien belegt. 5cx-MEPA konnte hierbei erstmalig und mit Konzentrationen deutlich über denen der anderen beiden Biomarker (5OH- und 5oxo-MEHA) im menschlichen Urin nachgewiesen werden. Die höchste Empfindlichkeit von 5cx-MEPA als Expositionsbiomaker wurde in den Pilotpopulationen aus Deutschland und Brasilien, welche keiner bekannten DEHA-Belastung ausgesetzt waren, bestätigt. Dort zeigte 5cx-MEPA eine DEHA Belastung

in 9% bzw. 43% der untersuchten Urine an, während die anderen beiden Metaboliten weniger häufig und in niedrigeren Konzentrationen gefunden wurden. Diese Pilotbetrachtungen demonstrieren somit die grundsätzliche Eignung der entwickelten Nachweismethode sowie der drei untersuchten Expositionsbiomarker (mit 5cx-MEPA als wichtigster Expositionsmarker) zur Erfassung der inneren Exposition gegenüber DEHA (auch zur Erfassung von Hintergrundexpositionen). Darüber hinaus bestätigten sie den Verzehr DEHA-belasteter Nahrung als relevante Expositionsquelle, aber auch eine durch unbekannte Expositionsquellen hervorgerufene messbare Belastung in der Allgemenbevölkerung. **(Kapitel 4)**

Um die im Urin gemessene Biomarkerkonzentration im Bezug zur Belastung beurteilen zu können, wurde der humane Metabolismus von DEHA nach oraler Verabreichung einer definierten Dosis an vier Probanden quantitativ untersucht. 5cx-MEPA wurde als spezifischer Hauptmetabolit (Mittelwert F_{UE}: 0,20%; Spanne: 0,17-0,24%) nachgewiesen. 5OH-MEHA (Mittelwert F_{UE}: 0,07%; Spanne: 0,03-0,10%) und 5oxo-MEHA (Mittelwert F_{UE}: 0,05%; Spanne: 0,01-0,06%) wurden mit geringeren Mengen über den Urin ausgeschieden. Diese Ausscheidungsverhältnisse bestätigen die Befunde aus den Pilotpopulationen. Die Eliminationskinetik der drei oxidativ modifizierten DEHA-Metaboliten zeichnet sich durch zwei Konzentrationsmaxima (t_{max1} = 1.5-2.3 h, t_{max2} = 3.8-6.4 h) sowie eine schnelle Elimination (98-100% der Gesamtmenge der ausgeschiedenen Metaboliten wurde 24 h nach oraler Dosis wiedergefunden) aus.

Basierend auf der gemessenen Konzentration 5cx-MEPA und dem abgeleiteten Konversionsfaktor wurde für die Pilotpopulationen aus Kapitel 4 der Daily Intake erstmals valide berechnet. Die höchste Aufnahmemenge DEHA wurde in einem Probanden erfasst, der durch die einmalige Nahrungsaufnahme gezielt gegenüber DEHA belastet wurde. Die resultierende berechnete Aufnahmemenge war einen Faktor 3 kleiner als der TDI von DEHA (0,3 mg/kg KG/Tag). **(Kapitel 5)**

Analog zu DEHA wurde auch für DINA ein weiterer neuer HBM-Ansatz entwickelt. Basierend auf den gewonnen Kenntnissen zu DEHA wurden spezifische Metaboliten mit Hydroxy-, Carbonyl- und Carboxylfunktion in der Alkylseitenkette als Biomarker postuliert und im Folgenden untersucht. Die besondere Herausforderung bei DINA war, trotz Isomerenpeaks der DINA-Metaboliten, eine hinreichend empfindliche analytische Methode zu entwickeln sodass Belastungen im Niedrigdosisbereich erfasst werden können. Mit einer ebenfalls neu entwickelten online-SPE-LC-MS/MS Methode, bei welcher kumulativ alle Metaboliten mit gleicher oxidativen Modifikation erfasst

werden, erfolgte erstmalig die quantitative Untersuchung des Human-Metabolismus von DINA nach oraler Verabreichung. In diesem Rahmen wurde die Anwendbarkeit der untersuchten Metaboliten als Biomarker zur Erfassung der Exposition gegenüber DINA qualifiziert und Konversionsfaktoren für die Beurteilung der gemessenen Biomarkerkonzentration hinsichtlich der Belastung und damit einhergehenden gesundheitlichen Risiken abgeleitet. Die hydroxylierten DINA-Metaboliten (OH-MINA) wurden als spezifische Hauptmetaboliten (Mittelwert F_{UE}: 0,022%; Spanne: 0,020-0,023%) identifiziert. Metaboliten mit Carbonylfunktion (oxo-MINA) und Carboxylfunktion (cx-MIOA) wiesen einen um Faktor 2 kleineren Dosisanteil auf. OH-MINA stellt somit den empfindlichsten Biomarker zur Erfassung der Exposition gegenüber DINA dar. Im Vergleich zu DEHA unterschied sich die oxidative Modifikation des spezifischen Hauptmetaboliten. Darüber hinaus wiesen die einfach modifizierten Metaboliten bei DINA einen geringeren Dosisanteil auf. Die Eliminationskinetiken der Metaboliten beider Adipate zeichnet sich durch eine schnelle Exkretion aus, wobei die Elimination von DINA nach 6 h abgeschlossen war, während der Hauptmetabolit von DEHA (5cx-MEPA) bis zu 32 h kontinuierlich in den Urinproben eines Probanden nach oraler Verabreichung nachzuweisen war. Für beide Adipate konnte ein Biomarker-Set bestehend aus Metaboliten mit Hydroxy-, Carbonyl- und Carboxylfunktion in der Alkylseitenkette indentifiziert werden. Die strukturellen Unterschiede beider Adipate in den Alkylseitenketten führen dabei jedoch zu Unterschieden im Dosisanteil und der Eliminationskinetik der jeweiligen Metaboliten. **(Kapitel 6)**

In Zukunft können die im Rahmen dieser Arbeit neu entwickelten HBM-Methoden in großangelegten Populationsstudien für eine Expositions- und Risikobewertung für DEHA und DINA angewandt werden. Wie für andere Weichmacher bereits durchgeführt, kann in archivierten Proben der Umweltprobenbank der zeitliche Verlauf der Belastung der Bevölkerung untersucht werden. Diese Untersuchungsergebnisse würden einen weiteren Einblick in den bis heute andauernden Umbruch im europäischen wie globalen Weichmachermarkt ermöglichen und einen möglichen Anstieg von DEHA aufgrund zunehmender Verwendung als DEHP-Substitut überprüfen. Des Weiteren können Messungen in aktuellen Bevölkerungsstudien (Umwelt-Surveys wie GerES V und VI) bevölkerungsrepräsentative Daten zur aktuellen Expositionssituation liefern und die Ableitung von Referenzwerten (RV95) ermöglichen.

Gleichzeitig wurde mit der quantitativen Beschreibung des Human-Metabolismus die Basis für die Ableitung eines HBM-I-Wertes geschaffen. Die Ableitung eines HBM-I-Wertes durch die Kommission Human-Biomonitrinmg des Umweltbundesamtes steht jedoch für beide Substanzen noch aus. Bis dahin kann die Rückrechnung auf die individuell aufgenommene tägliche Dosis zur Risikobeurteilung über den Vergleich mit toxikologisch abgeleiteten Grenzwerten (bspw. TDI bei DEHA) genutz werden.

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Anhang

Publikationsliste

Teilergebnisse der vorliegenden Arbeit wurden in Form von Publikationen in Fachzeitschriften veröffentlicht und als Tagungsbeiträge auf nationalen und internationalen Tagungen vorgestellt.

Veröffentlichungen in Fachzeitschriften:

- Nehring A, Bury D, Kling H-W, Weiss T, Brüning T, Koch HM (2019) Determination of human urinary metabolites of the plasticizer di(2-ethylhexyl) adipate (DEHA) by online-SPE-HPLC-MS/MS. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1124:239–246. doi: 10.1016/j.jchromb.2019.06.019.

- Nehring A, Bury D, Ringbeck B, Kling H-W, Otter R, Weiss T, Brüning T, Koch HM (2020) Metabolism and urinary excretion kinetics of di(2-ethylhexyl) adipate (DEHA) in four human volunteers after a single oral dose. Toxicol. Lett. 321:95–102. doi: 10.1016/j.toxlet.2019.12.006

- Gotthardt A, Bury D, Kling H-W, Otter R, Weiss T, Brüning T, Koch HM (2021) Quantitative investigation of the urinary excretion of three specific monoester metabolites of the plasticizer diisononyl adipate (DINA). EXCLI Journal 20:412-425. doi: 10.17179/excli2021-3360

Tagungsbeiträge

- Nehring A, Bury D, Otter R, Kling H-W, Weiss T, Brüning T, Koch HM (2018) Human Urinary Biomarkers of Exposure to the Plasicizer DEHA. Posterbeitrag zum 28th Annual Meeting of the International Society of Exposure Science joint with the International Society for Environmental Epidemiology (ISES-ISEE), 26.-30. August 2018 in Ottawa.

- Nehring A, Bury D, Otter R, Kling H-W, Weiss T, Brüning T, Koch HM (2018) Exposure to Phthalate Plasticizer Alternatives: Determination of DEHA Biomarkers in Human Urine. Posterbeitrag zum 1st European Exposure Science Strategy Workshop, 19.-20. Juni 2018 in Dortmund.

- Nehring A, Bury D, Ringbeck B, Otter R, Kling H-W, Weiss T, Brüning T, Koch HM (2019) Human Biomonitoring of the Alternative Plasticizer Di(2-ethylhexyl) adipate. Vortrag zum 11th International Symposium on Biological Monitoring in Occupational and Environmental Health, 28.-30. August 2019 in Leuven, Belgien.

Lebenslauf

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.