European Council for
Plasticisers and Intermediates
COMMITTED TO THE SCIENCE OF SAFETY

March 29, 2011

Office of the Secretary
Consumer Product Safety Commission
4330 East-West Highway
Bethesda, Maryland 2081
USA

Fao: Office of the Secretary - Consumer Product Safety Commission

Dear Sir or Madam,

The European Council of Plasticisers and Intermediates is providing this package of information in support of the ongoing work of the Consumer Product Safety Commission (CPSC) Chronic Hazard Advisory Panel (CHAP) relating to hazard, exposure, and risk information on phthalates and phthalate substitutes.

The list of attachments below has been provided to the European Commission, DG SANCO (Public Health) as part of the ongoing review by the EU Scientific Committees on Professor Kortenkamp's State of the Art Report on the Toxicity of Mixtures:

Cover letter sent to DG SANCO on September 3, 2010 Attachment I - Definition of low molecular weight (LMW) and high molecular weight (HMW) phthalates Attachment II - Review of scientific data on DINP and DIDP Attachment III - Scientific studies on phthalates relevant to LMW and HMW phthalates Attachment IV - Answers to six questions as they pertain to phthalates Attachment V - Ravenzwaay et al

The EU Scientific Committee review on Professor Kortenkamp's State of the Art Report on the Toxicity of Mixtures is expected in June 2011. Minutes from a recent EU Scientific Committee Health and Environmental Risks Working Group on Mixtures are included below and state the following:

"- In the answers of question 1, it should be clearly indicated that this WG does not agree with the evidence presented in The State of the Art report about the effects of low-dose concentrations because this is the key issue and the focus of attention by the interest groups (NGOs and industry)."

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Question 1. posed by the European Commission to the Scientific Committees is: Is there clear scientific evidence that when living organisms are exposed to a number of different chemical substances, that these substances may act jointly in a way (addition, antagonism, potentiation, synergies etc) that affects the overall level of toxicity?

If there are any questions relating to this information please do not hesitate to contact me.

Yours sincerely,

[redacted]

Maggie Saykali

Sector group manager
ECPI - European Council for Plasticisers and Intermediates

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Attachments:

- Letter Re Mixtures of chemicals – Call for information
- Definitions of LMW phthalates and HMW phthalates
- Review of scientific data on DINP and DIDP
- Scientific studies on phthalates relevant to mixtures toxicity and implications for cumulative risk assessment
- Answers to the six questions as they pertain to phthalate esters
- Ravenzwaay et al. (2010), Toxicology Letters 198 (2010) 159-170
- European Commission Minutes of the 5th Working group meeting on Toxicity of Chemical Mixtures

European Council for Plasticisers and Intermediates

COMMITTED TO THE SCIENCE OF SAFETY

Brussels, September 6th, 2010

Dear Sir or Madam,

Re: Mixtures of chemicals - Call for information

The European Council of Plasticisers and Intermediates, representing the major European Plasticiser Manufacturers would like to submit information on mixtures of chemicals relevant to a major group of plasticisers, namely phthalate esters. While it is understood that the Scientific Committee review is looking broadly at mixtures, several mixtures research studies and mixtures risk assessments have been conducted on "phthalates" and published in the scientific literature and by recognized agencies. It is important that the full scientific information on these studies is available in the context of the broad review on mixtures.

ECPI is providing this information to support the above referenced scientific assessment and to help ensure that transparent and robust information is provided with respect to phthalate esters.

In this context it is important to be aware that there are significant differences between Low Molecular Weight (LMW) and High Molecular Weight (HMW) Phthalates. Attachment I provides definitions for LMW and HMW phthalates. LMW phthalates are reproductive agents as shown by laboratory animal studies, are classified as Category 1B (CLP Regulation) Reproductive Agents and research suggests that for some reproductive effects they may act via a common endocrine mechanism. As such, LMW were included in the REACH Candidate List and will be subject to Authorisation.

Based on extensive data and evaluations HMW phthalates are not classified as reproductive agents and are not endocrine disrupters. Attachment II to this letter provides a summary of the key peer-reviewed studies relevant to HMW phthalates and which lead to the conclusion that HMW phthalates are not endocrine disrupters. Attachment II is currently being updated with a review of all relevant studies for LMW phthalates and HMW phthalates versus the OECD Endocrine Framework and an ECPI Technical Report will be issued in due course.

"Phthalates" are increasingly being cited as substances which have shown and/or which may have the potential for mixture effects in laboratory studies and for which mixtures risk assessments should be conducted. While the term "phthalates" is used very often in the literature, the research papers are reporting work on the classified LMW phthalates and typically not on HMW phthalates.

Research studies have shown that LMW phthalates may have the potential for additivity for the reported reproductive adverse effects in laboratory animal studies, and further work looking at combined effects and risk assessments may be appropriate for LMW phthalates. Since HMW phthalates do not have the same hazardous properties as LMW phthalates and are not reproductive agents it is not scientifically justified to propose a mixtures risk assessment for HMW phthalates, and further mixtures work with HMW phthalates is

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therefore of low priority. Attachment III to this letter provides a review of all the relevant studies and publications relevant to mixtures data for LMW phthalates and HMW phthalates.

Attachment IV provides answers with respect to LMW phthalates and HMW phthalates to the key questions to be addressed by the Scientific Committee review.

Attachment V shows that even when dealing with a mixture of only 2 compounds it may not be possible to just add up the effects (changes is metabolomic profile) of the single components: Simultaneous exposure to high dose levels of DEHP (up to 3000 ppm) and DBP (up to 7000 ppm) resulted in a metabolomic profile that was different compared to the individual compounds. A quantitative statistical analysis of the data revealed that the effect of combined treatment on the metabolites was less than additive.

If there are any questions on the above information or any other way in which ECPI can support the Scientific Committee review please do not hesitate to contact me.

Yours sincerely,

Maggie Saykali

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Attachment V	Ravenzwaay et al. (2010), Toxicology Letters 198 (2010) 159-170

Attachment I – Definition of LMW phthalates and HMW phthalates

Phthalate esters are a diverse group of substances produced by the reaction of phthalic anhydride with aliphatic and aromatic alcohols to produce di-esters. Certain phthalate esters are used extensively as PVC plasticisers and also in rubber products, paints and coatings and printing inks. Certain specific phthalates (DMP, DEP) are used in cosmetics and toiletries. Since the term "phthalates" constitutes a broad class of chemicals with a wide range of physical and chemical properties, it follows that not all phthalates are toxicologically equivalent. The major commercial products used in PVC, rubber products, paints, coatings and printing inks, can be divided into two main groups – Low Molecular Weight Phthalates (LMW) and High Molecular Weight Phthalates (HMW).

Low Molecular Weight (LMW) Phthalates

Low molecular weight (LMW) phthalates are those with alkyl side chains of C4 – C8 total carbon number. The carbon backbones in the side-chains of LMW phthalates are C3 – C6. Members of this group include Di(2-ethylhexyl) Phthalate (DEHP, also known commonly as DOP), Di-Butyl Phthalate (DBP), Butyl Benzyl Phthalate (BBP), Di-IsoButyl Phthalate (DIBP), Di-IsoPentyl Phthalate (DIPP), Di-IsoHeptyl Phthalate (DIHP). These LMW phthalates are classified as reproductive and developmental toxins (Category 1B under the UN Globally Harmonized System and the EU Classification, Labeling and Packaging Regulation) due to the significant adverse health effects observed in rodent studies.

Note: The very low molecular weight phthalates (VLMW) such as Di-Methyl Phthalate (DMP – carbon side chains of one carbon) and Di-Ethyl Phthalate (DEP – carbon side chains of two carbons) used in cosmetics and toiletries are not classified for reproductive effects.

High Molecular Weight (HMW) Phthalates

High molecular weight (**HMW**) phthalates are those with **carbon side chains of C9 and greater total carbon (typically to C13). The carbon backbones in the side-chains of HMW phthalates are C7 and greater.** Members of this group include Di-IsoNonyl Phthalate (DINP), Di-IsoDecyl Phthalate (DIDP) and Di-(2-PropylHeptyl) Phthalate (DPHP). Based on comprehensive data and evaluations these substances are NOT classified as reproductive and developmental toxins as they do not produce adverse reproductive effects.

In summary:

	Total Carbon in alkyl side chains	Carbon backbone in alkyl side chains	Classification CLP Regulation
LMW Phthalates	C4 – C8	C3 – C6	Category 1B Repro
HMW Phthalates	C9 – C13	C7 – C13	Not Classified

Comments relevant to uses and potential exposure to LMW phthalates and HMW phthalates

- All phthalates used in PVC applications are physically bound within the polymer matrix and only very severe conditions (e.g. solvent extraction) will lead to significant migration from the PVC. In practice migration occurs only at a very low level – if this was not the case then many everyday articles (e.g. electrical cables) would not function as intended. Migration is reduced to even lower levels with HMW phthalates. The fact that the phthalate plasticisers are not covalently bound within the polymer matrix and can be extracted by strong solvents contributes to the efficient recycling of the plasticizer and the PVC resin.
- DEHP (LMW) is used in PVC medical applications.
- DEHP, DBP and BBP (and other LMW phthalates classified as Category 1B (CLP Regulation) reproductive agents) are restricted from use in cosmetics by the EU Cosmetics Directive and hence exposure from this use to LMW classified phthalates is unlikely.
- DEHP, DBP and BBP (LMW) are restricted from use in all toys and childcare articles and hence exposure from this source is eliminated.
- Non-classified HMW phthalates have replaced classified LMW phthalates to a significant degree in general purpose applications such as wire and cable, flooring, construction, automotive applications.

Review of Scientific Data on Di-isononyl Phthalate (DINP) and Di-isodecyl phthalate (DIDP) demonstrating that neither are endocrine disrupters

(CAS No. 68515-48-0 / EINECS No. 271-090-9, 1,2-benzenedicarboxylic acid, di-C8-10-branched alkyl esters, C9-rich; CAS No. 28553-12-0 / EINECS No. 249-079-5 di-isononyl phthalate; and CAS No. 68515-49-1 / EINECS No. 271-091-4 1,2-Benzenedicarboxylic acid, di-C9-11-branched alkyl esters, C10-rich, CAS No. 26761-40-0 / EINECS No. 247-977-1 di-isodecyl phthalate)

European Council for Plasticisers and Intermediates
Technical Report 2009-1001-DINP and DIDP
October 2009 (updated November 2009)

V4.0 – November 10, 2009

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Summary Conclusions

- **DINP and DIDP are not endocrine disrupters**
- **DINP and DIDP are two of the most widely studied and evaluated chemical substances in the world**
    - Studies and evaluations have been conducted intensively and thoroughly over the last 30 years, with demonstrated safe use for 50 years.
- **DINP and DIDP are not endocrine disrupters as defined by Weybridge, IPCS and the draft REACH Guidance**
    - The definitions for endocrine effects (Weybridge definition, International Programme for Chemical Safety [IPCS], draft REACH Guidance) require evidence of adverse health effects in intact organisms, or progeny, or subpopulations mediated via an effect on functioning of the endocrine system.
    - DINP and DIDP have shown no evidence of endocrine related adverse health effects in intact organisms, chronic and sub-chronic toxicology studies, and endocrine screening studies.
    - DINP and DIDP have been found not to cause adverse effects on reproduction in two-generation rodent studies. OECD considers the two-generation study to be the most rigorous for testing and assessing effects of endocrine-disrupting chemicals on the reproductive system.
    - Limited human epidemiological studies also have been conducted on the association of endocrine effects and phthalate metabolite levels in breast milk and urine. These studies, as well, do not show any evidence of an association between DINP and DIDP exposure and endocrine disruption.
- **The EU Risk Assessments for DINP and DIDP evaluated all of the above studies and concluded that there is little concern with regard to potential endocrine effects.**
    - DINP and DIDP have been assessed by the relevant EU authorities for classification and labeling for reproductive effects, with the conclusion that classification and labeling for such effects is not required.
    - In addition the EU authorities have concluded that DINP and DIDP are not considered hazardous under any of the other EU classification categories.
- **For these reasons DINP and DIDP do not meet internationally accepted definitions for endocrine disruption, including those in the REACH Guidance**

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DINP and DIDP are not Endocrine Disrupters

The main body of the paper is divided into eight sections:

1. Two generation reproductive studies on DINP and DIDP and the EU Risk Assessment conclusions
2. Status of endocrine testing guidelines
3. Lack of oestrogenic activity for DINP and DIDP
4. Studies on anti-androgenic effects
5. Human data on endocrine effects and DINP/DIDP
6. Definitions for endocrine disrupters
7. Conclusions
8. References

   Appendix – The endocrine system

1. Two generation reproductive studies on DINP and DIDP and the EU Risk Assessment Conclusions

DINP and DIDP do not cause reproductive effects in rodent two generation reproductive toxicity studies. These studies provide a comprehensive basis on which to evaluate reproductive and developmental effects, including anti-androgenic effects in male rat pups. As such, DINP and DIDP were not classified as reproductive toxicants as part of the EU Risk Assessments. There are reports that DINP minimally modulated the androgenic endocrine system in developing rats (Gray et al, 2000), but only at doses that are well above relevant exposures, and this modulation did not produce adverse effects. These studies were considered in the recently published EU risk assessments for DINP and it was concluded that the effects are of little concern. The EU Scientific Committee for Toxicology, Ecotoxicology and the Environment (CSTEE) evaluated the endocrine disrupting properties of DINP to be "very low"; CSTEE took into account the slight effects seen at very high dose levels.

2. Status of endocrine testing guidelines

There is currently much effort focused on the development of validated in vivo and in vitro test methods for identifying endocrine disrupting substances notably under the OECD Test Guidelines programme, and in the United States and Japan. However, there are currently no internationally agreed methodologies or criteria available for the evaluation and confirmation of endocrine effects. OECD has developed a conceptual framework for the testing and assessment of potential endocrine disrupters, and included at the highest level (Level 5) is the 2-generation reproductive study. Inclusion at Level 5 underlines the importance of this test in defining endocrine disrupters.

3. Lack of oestrogenic activity for DINP and DIDP

Initially, discussion of endocrine modulation and phthalate esters focused on oestrogenic effects. Some phthalate esters were found to bind to the oestrogen receptor; however,

the binding was later determined to be non specific and did not lead to an activation of the oestrogen receptor. Lack of oestrogenic activity of phthalates was confirmed in cellular and whole animal assays for oestrogenic activity. In this analysis, both DINP and DIDP were evaluated and found to be without effect. As such, it is now accepted in the scientific community that DINP and DIDP are not oestrogenic nor anti-oestrogenic (Zacharewski et al., 1999).

4. Studies on anti-androgenic effects

Some oral gavage studies on DINP (Gray et al., 2000; Borch et al., 2004) have shown effects in male rat offspring at a single very high dose level, which is well above relevant exposure levels. The effects produced were of low incidence (observed in few pups) and were of low severity (reduced testosterone synthesis, areola or nipple retention). It is possible that these effects are specific to rats e.g. nipple retention is normal in humans but not normal in male rats. Further research is ongoing to understand the mechanism of these effects and whether they are rodent specific. It should be noted that the two generation reproductive toxicity study is the definitive study in this respect and no reproductive effects were seen in the DINP two-generation study (Waterman et al., 2000). To date, no data are available that indicate DIDP produces anti-androgenic effects in male rats, and no reproductive deficits were observed in two generation studies in rats with DIDP.

5. Human data on endocrine effects and DINP/DIDP

Recent research studies have evaluated relationships between fetal and neonatal exposure to phthalates and markers of endocrine mediated reproductive toxicity in humans. Swan et al (2005) investigated the association between metabolites of several phthalates in urine and anti-androgenic effects in young boys. Metabolites of DINP and DIDP were not analysed in this study. Serious flaws were identified in the study design and statistical analyses employed. Because of these flaws EPA decided not to utilize these studies for hazard and risk assessment.

In a separate study (Main et al, 2006), reported a statistically significant association between levels of DINP metabolites in breast milk of mothers and raised blood levels of leutinizing hormone in infant males. When converting the ratios reported to levels of luteinizing hormone, the levels are actually within normal limits for infants. Other measures of anti-androgenicity, such as reduced testosterone levels were not observed. In fact there was even a trend toward increased testosterone levels which would clearly not support an anti-androgenic effect for DINP. Further, there is still significant scientific uncertainty surrounding the significance of changes in the endpoints examined in the above studies. In addition changes in hormone levels alone are insufficient to conclude on endocrine disrupting properties. Therefore the studies should be considered as scientific research to generate research hypotheses but should not be used for safety evaluation purposes.

6. Definitions for endocrine disrupters

Endocrine disruption has been identified by the European Union (EU) as a criterion to identify substances of equivalent concern under the REACH regulation (Article 57 (f)). Substances of equivalent concern are "substances, such as those having endocrine disruption properties...for which there is scientific evidence of probable serious effects to

humans or the environment which gives rise to an equivalent level of concern to those of the substances listed in points (a) to (e) [Category 2 carcinogens, mutagens, or reproductive agents (CMR), or persistent, bioaccumulative and toxic substances (PBT), or very persistence and very bioaccumulative (vPvB)." The REACH guidance document ("Guidance for the preparation of an Annex XV dossier on the identification of substances of very high concern") provides a definition of an endocrine disrupter and recommends that given the complexities of the possible mechanisms and effects of endocrine active substances then a weight of evidence approach is needed.

Several definitions of endocrine disruption exist already today. One definition was developed in the late 1990s at an EU sponsored workshop on endocrine disruption in Weybridge, UK. The Weybridge Definition states:

> *"An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in the endocrine function".*

More recently, the International Programme for Chemical Safety (IPCS) has modified this definition slightly but still with the same overall meaning. The IPCS definition states:

> *"Endocrine disrupters have been defined as exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism or its progeny secondary to changes in endocrine function."*

This definition is referenced in the EU "Community Strategy for endocrine disrupters" (COM 1999 (706) final).

Further, the IPCS identifies three possible pathways for interference with the endocrine system

- By mimicking the action of a naturally-produced hormone such as oestrogen or testosterone, and thereby triggering similar chemical reactions in the body,
- By blocking the receptors in cells receiving the hormones (hormone receptors), thereby preventing the action of normal hormones; or
- By affecting the synthesis, transport, metabolism and excretion of hormones, thus altering the concentration of natural hormones.

The REACH guidance ("Guidance for the preparation of an Annex XV dossier on the identification of substances of very high concern") provides a definition of an endocrine disrupter which is almost identical to the above IPCS definition:

> *"An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.*

Based on the three definitions, adverse health effects must be produced as a consequence of endocrine disruption for the conclusion that a substance is an endocrine disrupter. From this it can be concluded that changes in hormone levels by themselves are not sufficient for classification of a chemical as an endocrine disrupter. Hormone levels are changing all the time due to normal cycles and due to external factors e.g. the menstrual cycle in women e.g. consumption of sugar causes insulin levels to rise. Studies which show an increase in hormone levels alone would not be sufficient to classify a substance as an endocrine disrupter, according to the above definitions.

7. Conclusion

- DINP and DIDP are not endocrine disrupters as defined by Weybridge, IPCS and the draft REACH Guidance.

8. References

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CSTEE (2001). **Opinion** on the results of the Risk Assessment of: 1,2-Benzenedicarboxylic acid, di-C8-10-branched alkyl esters, C9-rich and di-"isononyl" phthalate - CAS No.: 68515-48-0 and CAS No.: 28553-12-0 - EINECS No.: 271-090-9 and EINECS No.: 249-079-5. Report version (Human Health Effects): Final report, May 2001carried out in the framework of Council Regulation (EEC) 793/93 on the evaluation and control of the risks of existing substances. Opinion expressed at the 27th CSTEE plenary meeting, Brussels, 30 October 2001.

European Union Risk Assessment Report: DINP, European Chemicals Bureau, European Commission Joint Research Centre, 2nd Priority List, Volume 35 (2003). Full report available at the European Chemicals Bureau website:
www.ecb.jrc.it/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/dinpreport046.pdf

European Union Risk Assessment Report: DIDP, European Chemicals Bureau, European Commission Joint Research Centre, 2nd Priority List, Volume 36 (2003).Full report available at the European Chemicals Bureau website:
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Waterman, S, Keller, L, Trimmer, G, Freeman, J, Nikiforov, A, Harris, S, Nicholich, M, and McKee R. (2000). Two generation reproduction study in rats given di-isononyl phthalate in the diet. Reproductive Toxicology 14: 21-36.

Zacharewski, T.R., Meek, M.D., Clemons, J.H., Wu, Z.F., Fielden, M.R., Matthews, J.B. (1998) Examination of the in Vitro and in Vivo Estrogenic Activities of eight commercial phthalate esters. Toxicol. Sci. 46, 282 - 293

Note:

This paper addresses mammalian endocrine disruption. A separate ECPI paper is being prepared which will address endocrine disruption and lack of effects on fish and aquatic organisms.

Information on DINP and the fact it is not an endocrine disrupter is publicly available on the DINP Information Centre website: http://www.dinp-facts.com/endocrine

Appendix – The endocrine system

The endocrine system is actually comprised of several discrete systems that are important in regulation of growth, metabolism, development, fluid and mineral balance and reproduction (see table below). Endocrine systems can be comprised of one or several glands that synthesize and secrete substances called hormones into the blood stream. Individual endocrine glands synthesize, store and secrete hormones into the blood streams; some glands secrete more than one hormone, however, these individual hormones are synthesized by discrete cell types within these glands. Once in the blood stream, hormones interact with sites separate from the endocrine gland to produce a desired change in body function.

Brief overview of Major Endocrine Systems			
Body Function	Function	Gland(s)	Hormone(s)
Metabolism	Control glucose levels	Pancreas	Insulin
Growth	Increase size	Pituitary	Growth Hormone
Metabolism	Control metabolic rate	Hypothalamus Pituitary Thyroid	Thyrotropin Releasing hormone Thyroid Stimulating hormone Thyroxine
Mineral Balance	Control of Calcium levels	Parathyroid gland	Parathyroid hormone Calcitonin Vitamin D
Reproduction (female)	Control of menstrual cycle	Hypothalamus Pituitary Ovary (Follicular cells)	Estrogen Progesterone Leutenizing hormone Follicle Stimulating hormone Gonadotropin releasing hormone
Reproduction (male)	Production of sperm	Hypothalamus Pituitary Testes (Leydig cells, Sertoli cells)	Testosterone Leutenizing hormone Follicle Stimulating hormone Inhibin Gonadotropin releasing hormone

All endocrine systems monitor and respond to alterations of the environment within the body. When a stimulus is detected, hormone is released until the stimulus is removed, creating a feedback loop. Some endocrine systems have a simple feedback loop involving one gland and one hormone (i.e., pancreatic release of insulin in response to glucose). For others, the control and release of hormones can be quite complex involving several glands and several hormones (e.g. thyroid hormone control of metabolic rate).

In addition to maintenance of homeostasis, endocrine systems play an important role in normal growth and development. There are critical periods of development in which deficit of hormones results in abnormal development with serious consequences for health. For example absence of thyroid hormone during youth can result in cretinism, resulting in stunted growth and below average intelligence. Additionally, development of the reproductive system and both primary and secondary sexual characteristics requires the synthesis and release of the appropriate hormones at critical times.

Although endocrine systems have been markedly conserved through evolution, there are notable species differences in the operation and maintenance of these systems. These differences are due to the lack of auxiliary structures supporting the endocrine system, or due to differences in how the system as a whole functions. As an example of the former, rats lack thyroid hormone binding globulin, making them more susceptible to perturbations in thyroid hormone levels. For the latter, control of the female reproductive cycle is radically different between rodents and primates. In rodents female rats undergo an oestrous cycle, whereas primates go through a menstrual cycle. The same hormones are used to control different female reproductive cycles.

Attachment III
Scientific studies on phthalates relevant to mixtures toxicity and implications for cumulative risk assessment

Introduction

Phthalate esters are a diverse group of substances produced by the reaction of phthalic anhydride with aliphatic and aromatic alcohols to produce di-esters. Phthalate esters are used extensively as PVC plasticisers and also in rubber products, paints and coatings and printing inks. Certain specific phthalates (DMP, DEP) are used in cosmetics and toiletries. Since the term "phthalates" constitutes a broad class of chemicals with a wide range of physical and chemical properties, it follows that not all phthalates are toxicologically equivalent. The majority of phthalates used in commercial products (PVC, rubber products, paints, coatings and printing inks) can be divided into two main groups – Low Molecular Weight Phthalates (LMW) and High Molecular Weight Phthalates (HMW).

As a group, phthalates possess varied toxicological properties. A distinct area of differentiation is the reproductive and developmental effects observed in rodents for LMW phthalates, but not seen with HMW phthalates. These differences in reproductive effects between LMW and HMW phthalates are reviewed by Fabjan et al. (2006).

Research has examined phthalates, primarily LMW phthalates, in mixtures experiments to determine if adverse reproductive and developmental effects are additive in nature (NRC, 2008; Rider *et al.*, 2009).

Low Molecular Weight (LMW) Phthalates

Low molecular weight (LMW) phthalates are those with alkyl side chains of C4 – C8 total carbon number. The carbon backbones in the side-chains of LMW phthalates are C3 – C6 with methyl or ethyl sidechains to make up the total carbon number. Members of this group include Di(2-ethylhexyl) Phthalate (DEHP, also known commonly as DOP), Di-Butyl Phthalate (DBP), Butyl Benzyl Phthalate (BBP), Di-IsoButyl Phthalate (DIBP), Di-IsoPentyl Phthalate (DIPP), Di-IsoHeptyl Phthalate (DIHP).

Low molecular weight phthalates are classified as reproductive and developmental toxins (Category 1B under the UN Globally Harmonized System and the EU Classification, Labeling and Packaging Regulation) due to the significant adverse effects observed in rodent studies.[1] These effects include soft tissue and skeletal malformations in rodent fetuses, and toxicity to the testes (adult and fetal animals) and developing male rat reproductive tract during fetal and neonatal life stages in rats. The adverse effects observed in the male reproductive tract resultant from LMW phthalates include hypospadias, cryptorchidism, and alterations in male reproductive tract organ pathology which ultimately lead to decreased fertility. Reduced fertility has been observed in guideline two generation rat reproductive toxicity studies on LMW phthalates, particularly in the reproductive performance of subsequent generations (European Commission, 2004; European Commission, 2007; European Commission, 2008). It is likely that these classified LMW phthalates act in part via an endocrine mechanism and would therefore meet international definitions of an endocrine disrupter i.e. an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in the endocrine function (European Commission, 1996; International Programme on Chemical Safety, 2002). The endocrine related adverse health effects in this case are hypospadias, cryptorchidism and adverse male reproductive tract organ pathology observed in rats resulting in

[1] Di-Methyl Phthalate (DMP – carbon side chains of one carbon) and Di-Ethyl Phthalate (DEP – carbon side chains of two carbons), have carbon side chains less than 4 – 8 carbons and are not classified for reproductive effects.

reduced reproductive performance in subsequent generations observed in two generation reproductive toxicity studies.

It is important to note that some scientific papers and reviews often refer to endocrine effects of "phthalates" when in fact the data are specific to LMW phthalates and NOT HMW phthalates (Swan *et al.*, 2005; Lottrup *et al.*, 2006; Swan, 2008; Tanida *et al.*, 2009).

High Molecular Weight (HMW) Phthalates

High molecular weight (HMW) phthalates are those with carbon side chains of C9 and greater total carbon (typically to C13). The carbon backbones in the side-chains of HMW phthalates are C7 and greater. Members of this group include Di-Isononyl Phthalate (DINP), Di-Isodecyl Phthalate (DIDP) and Di-(2-propylheptyl) phthalate (DPHP).

A review of all the data on HMW phthalates (DINP, DIDP, and DPHP) has shown that these substances are not endocrine disrupters when assessed using the OECD Endocrine Framework. In particular, 2-generation reproduction studies (the highest tier study in the OECD Framework for Endocrine Assessment) in rats have shown no evidence of endocrine related effects: adverse histopathology, cryptorchidism, and hypospadias (European Commission, 2003a; European Commission, 2003b; NTP-CERHR, 2003a; NTP-CERHR, 2003b). Further, in marked contrast to LMW phthalates, there is no reduction in reproductive performance in subsequent generations in the two generation reproductive toxicity tests (Waterman et al, 2000; Hushka et al 2001). For DINP and DIDP these studies are referenced in the EU Risk Assessment Reports. OECD also conducted an HPV assessment of HMW phthalates including DINP, DIDP and DPHP and concluded these substances are of "low concern" for further work (OECD, 2004).

Based on comprehensive data and evaluations, HMW phthalates are NOT classified as reproductive and developmental toxins as they do not produce the adverse effects (fetal malformations, hypospadias, cryptorchidism and reduced fertility) observed with LMW phthalates. These substances do not meet the international definitions for an endocrine disrupter.

Mixture Studies on "phthalates"

Mixtures assessments have been conducted primarily with LMW phthalates to determine if effects on the developing male rat reproductive tract are additive in nature, specifically if they display dose addition.[2] A description of these studies is provided below. These studies have been cited by Kortenkamp et al. (2009) in a report produced for the European Commission entitled "State of the Art Report on Mixtures Toxicity".

Mixtures Studies with Classified LMW Phthalates

Howdeshell et al. (2007) examined a binary mixture of DBP and DEHP, two phthalates which are thought to have a common mode of action but have different active metabolites. Pregnant Sprague-Dawley rats (six dams per dose) were exposed to the phthalates during gestation days 14-18 at 500 mg/kg-d each, both singly and in combination. This dose was selected on the assumption that it would produce approximately half of the 50% incidence (EC_{50}) of epididymal agenesis. Male offspring were

[2] For mixtures of components that are determined to act through a common mode of action, the likelihood of toxicity associated with a mixture is determine by adding the doses of the components, where the concept of threshold is applied to the dose of the complete mixture, rather than to the doses of the individual components. The assumption for dose addition is that components are essentially toxicological "clones" of one another such that the relative proportions of each in a mixture are treated as dilutions of one another.

examined for a wide array of effects indicating maldevelopment of the male reproductive tract, including changes in fetal testosterone production, changes in anogenital distance, epididymal agenesis, retained nipples, gubernacular agenesis, hypospadias, and number of animals with malformations. The dose addition model generally predicted larger effects than the independent action model, although for some end points the two concepts predicted equal effects. However, experimental results indicated the responses generally agreed well with dose addition.

Howedeshell et al. (2008) evaluated suppression of fetal testosterone production at gestation day 18 following exposure of pregnant Sprague-Dawley rats to five phthalates separately and as a mixture. . In the first part of the study, pregnant dams were dosed at graded concentrations by gavage with BBP, DBP, DEHP, DIBP, and DPP to determine the effective dose which inhibited fetal testosterone production by 50% (ED_{50}). In the second portion of the study, the five phthalates were combined in a fixed ratio based upon their relative potencies such that each of the five phthalates would contribute equally to the reduction in testosterone. Results were modeled to an equation describing dose-addition. Over a large range of effect levels, the observed reductions in testosterone production agreed well with the responses predicted by the model, although there were small, statistically significant differences between the dose-addition prediction and the observed data.

Rider et al. (2008) conducted mixture experiments with the three phthalates BBP, DBP, and DEHP in combination with the antiandrogens vinclozolin, procymidone, linuron, and prochloraz. The mixture was given to pregnant rats with the aim of examining the male offspring for a variety of developmental effects typical of antiandrogens. The mixture components have varying modes of antiandrogenic action. Vinclozolin and procymidone are AR antagonists, BBP, DBP and DEHP suppress testosterone synthesis by altering activity and levels of enzyme critical to testosterone synthesis, and linuron and prochloraz exhibit a mixed mechanism of action both inhibiting steroid synthesis and blocking the steroid receptor. In calculating additivity expectations, the authors used historical data from their laboratory; however, the studies sometimes had dosing regimens that differed from those used in the mixture experiments. Data on the effects of some individual phthalates were not available. To bridge that data gap for the purpose of computing additivity expectations, it was assumed that the three phthalates were equipotent. Despite some uncertainty introduced by the equipotent assumption, dose addition gave predictions of combined effects of the mixed-mode antiandrogens that agreed better with the observed responses than did the expectations derived from independent action. For a number of end points, including seminal vesicle weights, epididymal agenesis, and nipple retention, there was reasonable agreement with dose addition. A statistical evaluation of the agreement between dose addition and experimental data was not provided by the study authors.

Ghisari and Bonefeld-Jorgensen (2009) reported a series of in vitro experiments examining the potential of BBP, DBP, DIDP, DINP, DEHP, tOP, CMP, 2,4-DCP, 2-PP resorcinol and DEHA to affect the thyroid hormone (TH) system and estrogen receptor (ER) function, alone and in combination. In order to obtain equipotent mixtures of the six plasticizers used in the mixture study (BBP, BPA, NP, tOP, CMP, and RES), the components were mixed on the basis of the single compounds no observed effect concentration, lowest observed effect concentration, and effective concentration 50%. When the mixture data was modeled, dose-additivity predicted the observed responses. DINP and DIDP, which were not included in the mixtures experiment, did not have any effect in the ER transactivation assay and only slight effect in the TH assay which occurred at the maximal dose tested. However, the utility of this information is questionable since phthalate diesters are rapidly metabolized to monoesters in humans (Silva *et al.*, 2006a; Koch *et al.*, 2007; Silva *et al.*, 2007). Conclusions drawn from diesters *in vitro* have no basis for extrapolation to *in vivo* systems.

Tanida et al. (2009) investigated the effects of fetal and neonatal exposure to three chemicals, bisphenol A, DEHP, and 2,3,7,8-tetrachlorodibenzo-p-dioxin, alone or in combination. Pregnant mouse dams were dosed with test material on gestation days 8-17. Neonates were dosed with test material on post natal days 3-7. Analysis occurred when pups were 2, 4, or 6 weeks of age. Brain samples were collected and examined immunohistochemically for tyrosine hydroxlyase (TH) and Fos-immunoreactive neurons as markers of dopamine and neuronal activation, respectively. Exposure to single substances results in changes in immunohistochemical signaling within midbrain doapminergic nuclei of mice, whereas such changes did not appear when the animals were exposed to the mixture.

It should be noted that these studies with LMW phthalates typically involve gavage dosing of large quantities of the test compound. This method of exposure is far removed from low level exposures to phthalates documented in the human population through biomonitoring studies (Silva *et al.*, 2004; Silva *et al.*, 2006b; Silva *et al.*, 2006c; Silva *et al.*, 2006d; Silva *et al.*, 2007; Wittassek *et al.*, 2007; Wittassek and Angerer, 2008; Wittassek *et al.*, 2010).

Mixtures Studies with Non-Classified HMW Phthalates

Borch et al (2004) examined hormonal effects in male rat fetuses exposed to DEHP, DINP, or a combination of the two. Thirty-two dams were dosed with either 300 mg DEHP/kg bodyweight per day, 750 mg DINP/kg bodyweight per day, or a combination of these doses. Male fetuses were examined on gestation day 21, and blood and testes were collected for hormone analysis. Reduction in *in vitro* testosterone synthesis was observed following DINP treatment, but in the absence of observation of adverse phenotypic outcomes. The authors report that a factorial statistical analysis revealed no statistically significant interaction between the effects of DEHP and DINP .

Ghisari and Bonefeld-Jorgensen (2009) reported a series of in vitro experiments examining the potential of BBP, DBP, DIDP, DINP, DEHP, tOP, CMP, 2,4-DCP, 2-PP resorcinol and DEHA to affect the thyroid hormone (TH) system and estrogen receptor (ER) function, alone and in combination. DINP and DIDP, which were not included in the mixtures experiment, did not have any effect in the ER transactivation assay and only slight effect in the TH assay which occurred at the maximal dose tested. However, the utility of this information is questionable since phthalate diesters are rapidly metabolized to monoesters in humans (Silva *et al.*, 2006a; Koch *et al.*, 2007; Silva *et al.*, 2007). Conclusions drawn from diesters *in vitro* have no basis for extrapolation to *in vivo* systems.

Cumulative Risk Assessment

Cumulative risk typically refers to the accumulation of risk from multiple chemical and/or non-chemical stressors. The interaction of these stressors may produce an additive, synergistic, or antagonistic response thus altering the individual risk of each stressor. This is different from aggregate risk assessment which refers to the sum of the risks resulting from exposures to the same chemical via multiple sources and multiple routes. The Danish EPA (2009) published a combined (aggregate) exposure risk assessment that included phthalates (DBP, DiBP, BBP, DEHP, DINP). As reported, daily ingestion specifically for DINP, from all sources, does not constitute a risk (page 226 of the report). In this study, reduced testicular weights in mice, was used as the endpoint of concern for DINP. When combining risk estimates for all chemicals, LMW phthalates (DBP and DEHP) significantly contributed to the risk characterization ratio whereas the DINP contribution was at least two-orders of magnitude lower.

Data generated from studies on the interactions of chemical mixtures can be used to inform cumulative risk assessments but do not indicate a cumulative risk actually exists. Results of these studies inform as to risk model selection (dose-addition, response addition, or independent action). Integration of the risk

model with estimates of exposure and hazard will define the cumulative risk. From the discussion above, the assumption of dose-addition appears to be supported by the mixtures studies with LMW phthalates. The assumption of dose addition as the basis for conducting a cumulative risk assessment for humans is highly conservative since dose-addition is assumed at levels below a threshold of response (Borgert *et al.*, 2004). Further, consideration for inclusion of chemicals in a cumulative risk assessment should be based on common adverse outcomes (i. e. reduced fertility) through a common mode of action.

The US National Research Council (NRC) published a report with the intention of answering two questions: should phthalates be subjected to a cumulative risk assessment and if so, how should it be conducted (National Research Council, 2008). On the basis of its review, the committee concluded that sufficient data are available to proceed with the cumulative risk assessment of phthalates. Additionally it was noted that addressing current data gaps in risk assessment would lead to greater refinement of a cumulative risk assessment and reduce uncertainty associated with any risk estimates.

Subsequent to the NRC report, two initial phthalate cumulative risk assessments have employed a hazard index approach where the critical "effect" included multiple developmental endpoints (Benson, 2009; Kortenkamp and Faust, 2010). Consistent with each individual chemical's ability to induce developmental and reproductive effects in rodents, the hazard quotients for DEHP and DBP were much larger than for DINP. These assessments indicate that DINP, a high molecular weight (HMW) phthalate, does not significantly contribute to the overall "phthalate" mixture toxicity and risk due to its low toxicity for the chosen endpoint and low exposure. Similar findings would be expected with the other HMW phthalates (DIDP and DPHP) due to the low estimated exposures and low potential to induce toxicity. As such their contribution to the overall risk of a mixture would not be significant. These findings further support the previously discussed observation that all phthalates are not toxicologically equivalent. LMW phthalates produce reproductive and developmental toxicity and when cumulative risk assessments address this endpoint, LMW phthalates significantly contribute to the overall assessment of risk. HMW phthalates (DINP, DIDP and DPHP) do not produce these effects and do not contribute to the overall risk presented by a mixture of phthalates.

Conclusions

Not all phthalates are the same; they are different toxicologically. LMW phthalates (DBP, BBP, DEHP) are classified as reproductive and developmental toxins due to the deleterious effects observed in laboratory animals and are considered endocrine disruptors. HMW phthalates (DINP and DIDP) are not classified and are not endocrine disruptors because they do not produce the adverse outcome, reduced fertility, in animal studies.

Mixtures studies are designed to test interactions (e.g. dose-addition and/or response-addition, synergy, antagonism) for mixtures of chemicals. Some mixtures studies indicate that LMW phthalates exhibit additivity of effect at doses near the observable effect range (i.e. high doses). In contrast, one study which tested a HMW phthalate (DINP) with LMW phthalate (DEHP) concluded no interaction for the endpoints examined.

Cumulative risk assessment serves the purpose to quantify the accumulation of risk from multiple chemical stressors that may interact. In initial cumulative risk assessments for phthalates, where the endpoint of concern is adverse effects on the developing male reproductive tract leading to an adverse outcome of reduced fertility, HMW phthalates do not contribute substantially to overall risk. This conclusion further supports the differentiation between LMW and HMW phthalates and questions the need to include HMW phthalates in cumulative risk assessments.

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Attachment IV – Answers to questions based on experience with LMW and HMW phthalate esters – the short answers below should be read in conjunction with the ECPI detailed input (letter plus Attachments I, II, and III.

The following table shows the six questions addressed by the Commission to the Scientific Committees and sets the categories for industry answers.

1) Is there clear scientific evidence that when living organisms are exposed to a number of different chemical substances, that these substances may act jointly in a way (addition, antagonism, potentiation, synergies etc) that affects the overall level of toxicity?

Contributor/company	Key findings	Reference/ Attachment
European Council for Plasticisers and Intermediates		See Attachments I, II, III to ECPI letter to DG SANCO

Mixtures assessments have been conducted primarily with LMW phthalates to determine if effects on the developing male rat reproductive tract are additive in nature, specifically if they display dose addition. A description of these studies is provided in Attachment III. These studies have been cited by Kortenkamp et al. (2009) in a report produced for the European Commission entitled "State of the Art Report on Mixtures Toxicity". In general, LMW phthalates do appear to show additivity of effect at doses near the observable effect range (i.e. high doses). However, dose addition is not a generalized phenomenon; there is no scientific basis for extrapolation to lower doses, levels to which humans are exposed.

2) If different chemical substances to which man/environment are exposed can be expected to act jointly in a way which affects their impact/toxicity on/for man and the environment, do the current assessment methods take proper account of these joint actions?

Contributor/company	Key findings	Reference/ Attachment
European Council for Plasticisers and Intermediates		See Attachments I, II, III to ECPI letter to DG SANCO

Current risk assessments on chemicals are highly conservative, giving confidence that additive or even rare synergistic effects are likely to be accounted for. Single chemical risk assessment (RA) does not underestimate risk. The practice is a realistic worst case such that exposures are estimated as being high compared to a low extrapolation from NOAEL levels to a highly conservative assessment. For intended or known joint exposures, mixtures toxicity can be relevant. Single chemical RA should still be the preferred model and use of mixture interactions only be applied on a specific needs basis. Such needs must be based on consistent criteria such as dose-response effect for key toxicological finding(s), mode of action, exposure estimations, metabolic interdependencies, species sensitivity etc.

The current system of regulating individual chemicals, while not intentionally designed to address cumulative effects, has nevertheless established approaches that will accommodate risks from mixtures.

As a group, phthalates possess varied toxicological properties. One distinct area of differentiation is the observed reproductive and developmental effects seen with low molecular weight (LMW) phthalates but not seen with high molecular weight (HMW). These differences limit the utility of certain endpoints with which to base a cumulative risk assessment. In general, endpoints should be chosen based on the commonality of the endpoint, availability of adequate published data, and toxicological concern.

If the contention is that additivity among phthalates leads to higher risks than calculated from individual phthalate assessments, then a quantitative method for determining the amount of an individual's risk that is missed by a chemical-by-chemical approach needs to be developed (Kamrin, 2009). Recent cumulative assessments examining reproductive and developmental endpoints of phthalate mixtures indicate that DINP is a minor contributor to the overall hazard index, an inherently conservative assessment. Kortenkamp and Faust (2010) report that DINP contributed only 0.11% to the cumulative toxicity of 15 chemicals while Benson (2009) demonstrated that DINP contributes 5% to the overall toxicity of 6 phthalates. This clearly indicates that the chemical by chemical approach would have identified and controlled the risk to the most toxic component (i.e LMW phthalates, specifically DEHP) of the mixture. If the risk posed by the most toxic component of the mixture is controlled or eliminated, then the risk posed by the overall mixture is no longer a concern.

3) Several models for the assessment of the mixture effects of chemicals already exist such as dose addition and independent action. What are the advantages and disadvantages of the different models and is there any particular model that could be considered as sufficiently robust to be used as a default option?

Contributor/company	Key findings	Reference/ Attachment
European Council for Plasticisers and Intermediates		See Attachments I, II, III to ECPI letter to DG SANCO

Dose-addition and independent action are two models proposed for use in mixtures risk assessment. Dose-addition is applied to mixtures of chemicals that have the same mode of action while the response addition (independent action) is utilized with mixtures containing chemicals with different modes of action (Lambert and Lipscomb, 2007).

In the dose-addition model, the likelihood of toxicity associated with a mixture is determined by adding the doses of the components, where the concept of threshold is applied to the dose of the complete mixture, rather than to the doses of the individual components as is done in response-addition.

The response-addition model, also referred to as independent- action model has been used to describe mixtures of chemicals with different mechanisms of action. Response addition also requires toxicity and exposure data for the mixture components. In contrast to dose addition, each individual mixture component response is determined directly from its dose-response relationship; these individual responses are summed across the mixture.

To guide the decision between which of the models to utilize, a critical health endpoint of concern must be identified from the available data. Next, a biologically plausible weight of evidence description of the key events in the postulated mode of action must be developed. With the development of an agreement on mode(s) of action and the degree of similarity or independence, a mixtures risk assessment could be performed utilizing one the of models described. Optimally, the choice of the mixtures risk assessment model should be based on the level of knowledge of the biologically relevant steps in the manifestation of toxicity.

It is important to recognize that the choice of using either dose-addition or independent action represents a default position, one to be replaced when data becomes available. For example, the application of dose-addition is likely to overestimate, but unlikely to underestimate mixture toxicity; thereby representing a conservation approach, where appropriate (McCarty and Borgert, 2006).

It is currently proposed that dose-addition should be the default approach (Kortenkamp *et al.*, 2009). The assumption is that dose-addition when observed at high doses will also be observed at low doses is false. Dose addition (non-independent action) may occur at high doses while response addition (independent action) occurs at low doses for some groups of chemicals. As stated by (Borgert *et al.*, 2004), dose addition may be a conservative assumption [for some effects] of chemicals when they are present at concentrations above their NOAELs, but that independence becomes more predictive when the concentrations of the component chemicals are below their individual NOAELs.

It is important to point out that the reason low dose mixtures may be less than additive is that the mode of action could be different below the NOAEL. Borgert *et al.*, (2004) also indicates that it is premature to assume dose addition for chemicals that appear to be mechanistically similar and to assume response addition models only for chemicals that appear to be mechanistically dissimilar. Because these simple models were developed for binary mixtures, their applicability to more complex mixtures is uncertain. Dose addition should be correlated with specific mechanistic features for particular toxic effects before the approach is generalized.

4)Given that it is unrealistic to assess every possible combination of chemical substances what is the most effective way to target resources on those combinations of chemicals that constitute the highest risk for man and the environment (tiered testing schemes, structurally similar groups of chemicals, chemicals with similar modes of action, chemicals acting on the same organ, chemicals in the same product group, chemicals shown by monitoring data to occur together in toxicologically significant concentrations etc)?

Contributor/company	Key findings	Reference/ Attachment
European Council for Plasticisers and Intermediates		See Attachments I, II, III to ECPI letter to DG SANCO

To guide the decision between which of the models to utilize (dose-addition or independent action), a critical health endpoint of concern must be identified from the available data (Lambert and Lipscomb, 2007). Next, a biologically plausible weight of

evidence description of the key events in the postulated mode of action must be developed. With the development of an agreement on mode(s) of action and the degree of similarity or independence, a mixtures risk assessment could be performed utilizing one the of models described. Optimally, the choice of the mixtures risk assessment model should be based on the level of knowledge of the biologically relevant steps in the manifestation of toxicity.

As a group, phthalates possess varied toxicological properties. One distinct area of differentiation is the observed reproductive and developmental effects seen with low molecular weight (LMW) phthalates but not seen with high molecular weight (HMW). These differences limit the utility of certain endpoints with which to base a cumulative risk assessment, and clearly show that a chemical family approach is not necessarily the correct approach to take. In general, endpoints should be chosen based on the commonality of the endpoint or mode of action, availability of adequate published data, and toxicological concern.

5) Where are the major knowledge gaps with regard to the assessment of the toxicity of chemical mixtures?

Contributor/company	Key findings	Reference/ Attachment
European Council for Plasticisers and Intermediates		See Attachments I, II, III to ECPI letter to DG SANCO

There are multiple knowledge gaps with regards to mixtures assessment, including:

1) There are no clear criteria for the extrapolation of combination effects from high doses to low doses. Mixtures studies are designed to test interactions (e.g. dose-addition and/or response-addition, synergy, antagonism) for mixtures of chemicals. Some mixtures studies indicate that LMW phthalates exhibit additivity of effect at doses near the observable effect range (i.e. high doses). In contrast, one study which tested a HMW phthalate (DINP) with LMW phthalate (DEHP) concluded no interaction for the endpoints examined. However, dose addition is not a generalized phenomenon; there is no scientific basis for extrapolation to lower doses, levels to which humans are exposed.

2) The utility of chemical potency in mixtures risk assessment is unclear. It would appear unnecessary to include chemicals of low potency/no effect. With respect to reproductive and development endpoints, HMW phthalates are toxicologically different than LMW phthalates. In Benson (2009) the low toxicity and low exposure led to minimal contribution of DINP to the overall toxicity of the mixture.

3) How should the mixture of concern be defined to ensure that the most meaningful interactions are addressed? Should this take into account common mode of action, mechanism of action, common toxicological endpoint?

4) How are extrapolations from animal data to humans accomplished for mixture effects?

6) Does current knowledge constitute a sufficiently solid foundation upon which to address the toxicity of chemical mixtures in a more systematic way in the context of EU chemicals' legislation?		
Contributor/company	**Key findings**	**Reference/ Attachment**
European Council for Plasticisers and Intermediates		See Attachments I, II, III to ECPI letter to DG SANCO

At this point in time, there are significant data gaps, uncertain methodologies, and lack of scientific validation for mixtures risk assessment. Substances should continue to be assessed individually to ensure all potential hazards are understood and to develop dose-response information to manage risks. Classes of chemicals should not be assessed simply on structural similarity unless there is sound rationale for read-across / category approaches. Where exposure information suggests possible combined exposures, it may be feasible to examine interactions of a substance within the context of its own risk assessment and to take into account structurally / mechanistically similar chemicals.

Relatively few studies have been conducted with phthalates to determine the potential interactions of phthalates in mixtures. In general, these studies have been conducted at very high doses and have not explored combination effects at doses that would be relevant to human exposure. A clear conclusion that HMW phthalates do not contribute to the risk of a mixture based on reproductive and developmental endpoints has been demonstrated (Benson, 2009; Kortenkamp and Faust, 2010). Current single chemical risk assessment methodologies for phthalates indicates that HMW phthalates (DINP/DIDP) pose insignificant risk based on low toxicities and low exposures.

As noted above it is unclear based on the phthalates experience whether a combined effects assessment would have changed the regulatory treatment of LMW phthalates. Individual substance hazard and risk assessments led to these substances being clearly identified as Category IB reproductive agents.
The knowledge base is available to a significant degree to identify and prioritize those substances for which a more in-depth evaluation including mixtures effects would be suitable. Based on clear criteria this should be possible: such criteria would include:

1. Degree of hazard (e.g. CMR substances)
2. Common target organs
3. Common mode of action
4. Significant human exposure and co-exposure to the different substances
5. Substances for which the margin between effect levels and exposure is narrow

In order to ensure a science based, targeted approach and efficient use of resources a mixtures hazard and risk assessment could be considered as part of the Evaluation and Authorisation phases of REACH where the above criteria are met.

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McCarty, L. S., and Borgert, C. J. (2006). Review of the toxicity of chemical mixtures: Theory, policy, and regulatory practice. *Regul Toxicol Pharmacol* **45**, 119-143.

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Toxicology Letters xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

The individual and combined metabolite profiles (metabolomics) of dibutylphthalate and di(2-ethylhexyl)phthalate following a 28-day dietary exposure in rats

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ARTICLE INFO

Article history:
Received 27 January 2010
Received in revised form 14 May 2010
Accepted 16 June 2010
Available online xxx

Keywords:
Metabolomics
Metabolite profiling
Dibutylphthalate
Di(2-ethylhexyl)phthalate
Mode of action
Combination toxicity

ABSTRACT

Metabolite profiles (metabolomics) of plasma samples of Wistar rats dosed with di(2-ethylhexyl)phthalate (DEHP – 3000 ppm) and dibutylphthalate (DBP – 150, 1000 and 7000 ppm) were individually determined in 28 days dietary studies. In addition, profiles of combined exposure to 3000 ppm DEHP and either 150, 1000 or 7000 ppm DBP were determined.

High dose levels induced more profound metabolite changes in males than in females for both compounds. At 150 ppm DBP (NOEL for toxicity) there were very few (<false positives rate), inconsistent changes, demonstrating a metabolomic NOEL. A part of the total metabolite profile was consistent with a pattern of changes indicative of peroxisome proliferation, confirmed by increased cyanide-insensitive Palmitoyl-CoA oxidation.

Simultaneous administration of 3000 ppm DEHP and 150 ppm DBP did not result in relevant changes when compared to the metabolite profile of 3000 ppm DEHP alone. Co-administration of 1000 ppm DBP induced marginal additional changes relative to the profile of 3000 ppm DEHP alone. Simultaneous exposure to high dose levels of DEHP and DBP resulted in a profile that was significantly different compared to the individual compounds. A quantitative statistical analysis of the data revealed that the effect of combined treatment on the metabolites was less than additive.

1. Introduction

The bulk of studies to assess the toxicity of chemicals deal with exposures to single compounds (Yang, 1994). Although toxicity studies of single compounds are important for obtaining basic toxicological information, humans are simultaneously exposed to a large number of chemicals.

With the possible exception of some specific mixtures, it is uncertain how the combined toxicity of these chemicals should be assessed or how combined toxicity should be taken into account in standard setting for the individual compounds. The main problems in the risk assessment of chemical mixtures are chemical interactions that hamper the prediction of the toxicity of the mixture. Since these interactions may occur at various end points in the toxicodynamic as well as the toxicokinetic phase, toxicologists have to deal with a vast scope of chemical interactions. In order to address questions of simultaneous exposure to multiple chemicals we designed a study to investigate the effects of two phthalates; dibutylphthalate (DBP) and di(2-ethylhexyl)phthalate (DEHP) dosed either individually or in combination applying endpoints of metabolomics parameters (Looser et al., 2005; Weckwerth and Morgenthal, 2005; Fernie et al., 2004).

Within the context of metabolite profiling (metabolomics), metabolites are defined as small endogenous compounds such as carbohydrates, amino acids, nucleic acids or fatty acids and their derivates resulting from biochemical pathways (Lindon et al., 2004). The use of sensitive LC–MS and GC–MS techniques offers the possibility to detect a broad range of metabolites and thus increases the chance of finding relevant biomarkers or patterns of change (Walk and Dostler, 2003). In addition some practical advantages (with blood as a matrix samples can be obtained by a less invasive method, no need to kill animals, time coarse analysis possible), metabolomics is usually more powerful (from a statistical perspective) in detecting robust effects compared to other "omics" technologies. In a typical transcriptomic/proteomic experiment the number of samples is usually rather small compared to the very high number of features (parameters such as RNA transcripts or proteins) which limits the ability of detecting unique effects by

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doi:10.1016/j.toxlet.2010.06.009

normalized to the median of reference samples which were derived from a pool formed from aliquots of all samples to account for inter- and intra-instrumental variation. Steroids hormones, catecholamines and their metabolites were measured by online SPE-LC-MS/MS (Solid phase extraction-LC-MS/MS) (Yamada et al., 2002). Absolute quantification was performed by means of stable isotope-labelled standards.

The methods applied resulted in 238 unique analytes for semi-quantitative analysis, 175 of which were chemically identified and 63 were unknown. Moreover, several hundred further analytes giving a fingerprint of the sample were included in the methods.

2.5. Further examinations

All animals were checked daily for any clinically abnormal signs and mortalities. Food consumption was determined on study days 6, 13, 20 and 27. Body weight was determined before the start of the administration period in order to randomize the animals and on study days 0, 3, 6, 13, 20 and 27.

At the end of the treatment period, the animals were sacrificed by decapitation under Isoflurane anesthesia. Organ weights of liver and testes were determined

Cyanide non-sensitive Palmitoyl-CoA Oxidation was measured in liver tissue homogenates on the Cobas Fara II analyzer, Roche, Germany, according to the method described by Lazarow (1981). The total protein concentration in the homogenates was measured with the Biuret method on the Hitachi 917 analyzer, Roche, Germany. The Palmitoyl CoA Oxidation values were related to the total protein levels.

2.6. Statistics

2.6.1. Metabolite profiling

The data were analysed by univariate and multivariate statistical methods. The sex- and day-stratified heteroscedastic t-test ("Welch test") was applied to log-transformed quantitive and semi-quantitive metabolite data to compare treated groups with respective controls. p-values, t-values, and ratios of corresponding group medians were collected as metabolic profiles and fed into a database (MetaMap® Tox). Metabolic profiles presented in this paper were developed applying 5% significance level and demanding statistical significance at least during two out of three time points.

2.6.2. Linear mixed-effects models

The study consists of a full factorial 4 × 2 × 2 × 3 repeated measure design: i.e. 4 – the dose levels which were used in the study, 2 – the treatment factors (DBP and DEHP), 2 – the sexes (male and female) and 3 – the sampling days (days 7, 14 and 28). A linear mixed-effects model was estimated for each of the 238 metabolites as response in order to quantify and test the phthalate main effects and their corresponding interactions while controlling for time and nuisance effects (intra- and inter-subject variance). Mixed-effects models were set up for both gender groups separately because results were assumed to be heterogeneous over sex.

Using short notation each metabolite "M" is represented by the following "random intercept" model equation:

$$M = \text{animal} + \text{DBP} + \text{DEHP} + \text{time} + \text{DBP} \times \text{DEHP} + \text{DBP} \times \text{time} + \text{DEHP} \times \text{time} + \text{epsilon}.$$

where animal stands for animal effects arising from inter-subject variance, DBP and DEHP denote treatment contrasts with respect to control animals, and the term time denotes day contrasts relative to day 7. The corresponding interactions are represented by the products of the main effects. In other words, the term "DBP × DEHP" tells how DEHP moderates the effect of DBP and vice versa. The random error term epsilon accounts for the unexplained variance after taking all other effects into account. This is a mixed-effects model because it simultaneously aims at estimating random (animal, epsilon) along with fixed effects (all other terms in the above equation). Statistical analysis was conducted using the statistical script language R (http://www.R-project.org).

The contrasts of interest were labeled using the following notation: (1) main treatment effects relative to the control group by "substance.dose" (dbp.150, dbp.1000, dbp.7000, dehp.3000), e.g. dbp.7000 = (DBP 7000 ppm group) – (Control group), and (2) interaction effects by "dehp.dbp.dose of DBP", e.g. dehp.dbp.7000 = interaction effect from combining dehp.3000 with dbp.7000 (constant dose 3000 of DEHP removed from the label as redundant).

Interaction contrasts quantify the non-additivity arising from combined phthalate administration. If the individual treatment effects and the interaction are of the same sign (all three positive or all three negative), then the combined effect is over-additive. If the sign of the individual treatment effects is different from the sign of the interaction, then the combined effect is under-additive.

2.6.3. Further examinations

For the Palmitoyl CoA Oxidation the values of the various groups were compared with the two-sided Wilcoxon test.

Table 2
Relative liver weights in the described 4 weeks Wistar rats study with administration of DBP and DEHP via the diet (two-sided Wilcoxon test: $^*p \leq 0.05$).

Relative liver weights	Males	Females
Control	2.192	2.332
DEHP 3000	2.999*	2.763*
DBP 150	2.197	2.181
DBP 1000	2.505*	2.385
DBP 7000	2.624*	2.700*
DEHP + DBP 150	2.919*	2.886*
DEHP + DBP 1000	2.827*	3.052*
DEHP + DBP 7000	3.109*	2.956*

3. Results

3.1. Clinical symptoms and clinical pathology

3.1.1. DBP

There were no clinical signs of toxicity in any of the treatment groups. Body weight development and food consumption were not affected in any of the treatment groups, with the possible exception of a 5% reduction (not statistically significant) in body weight in the 3000 ppm DEHP + 7000 ppm DBP males.

There was an increase of absolute liver weight in males and females in the 7000 ppm group. Relative liver weights were increased in high dose males and females, as well as in 1000 ppm males (see Table 2). There were no effects on absolute or relative testes weights in any of the DBP groups.

Treatment with 7000 ppm DBP resulted in a statistically significant ($p < 0.01$) increase in cyanide-insensitive Palmitoyl-CoA (P-CoA) oxidation levels in males only. In females there was a numerical increase after treatment with 7000 ppm and 1000 ppm DBP, which did not attain statistical significance. The values of the 150 ppm group in both sexes and those of the 1000 ppm males were virtually identical to those of the controls (see Fig. 1).

3.1.2. DEHP

There were no clinical signs of toxicity in males or females treated with 3000 ppm DEHP. Body weight development and food consumption were also not affected. There was an increase of absolute and relative liver weight in males and females treated with 3000 ppm DEHP (Table 2). There were no effects on absolute or relative testes weights (see Fig. 1). Treatment with 3000 ppm DEHP resulted in a statistically significant ($p < 0.01$) increase of P-CoA oxidation in males and females, which was most pronounced in males.

3.1.3. Combination DEHP and DBP

There were no clinical signs of toxicity in males or females in any of the combined DEHP and DBP treatment groups. Body weight development and food consumption were not affected by treatment. There was an increase of absolute and relative liver weight in males and females in all animals of the combined treatment groups. With increasing dose levels of DBP a slight further increase of liver weights could be noted. However, the highest liver weights in the female animals were detected in the animals receiving 3000 ppm DEHP and 1000 ppm DBP. Very weak increases of absolute and relative testes weights were observed (data not shown).

The combined treatment of 3000 ppm DEHP and 150 ppm DPB did not have an effect on P-CoA activity, relative to the activity observed with 3000 ppm DEHP alone. In the group receiving 3000 ppm DEHP and 1000 ppm DBP there was no effect on P-CoA activity in males (relative to 3000 ppm DEHP males). In females the mean P-CoA oxidation increased from 8.5 U/g protein (3000 ppm DEHP) to 11.1 U/g protein (3000 ppm DEHP + 1000 ppm DBP). This increase was statistically significant ($p < 0.05$). Com-

Please cite this article in press as: van Ravenzwaay, B., et al., The individual and combined metabolite profiles (metabolomics) of dibutylphthalate and di(2-ethylhexyl)phthalate following a 28-day dietary exposure in rats. Toxicol. Lett. (2010), doi:10.1016/j.toxlet.2010.06.009

Table 3
Metabolite level changes in *male* Wistar rats treated with 7000, 1000 and 150 ppm DBP for 4 weeks. Metabolite levels were measured at study days 7, 14 and 28. Metabolites are mentioned with at least at two of three study days increased (red marked) or decreased levels (yellow marked; WELCH *t*-test, $p \leq 0.05$) in one of the three mentioned dose groups. Figures represent relative changes of the median metabolite levels compared to controls.

Metabolite	DBP 7000 ppm			DBP 1000 ppm			DBP 150 ppm		
	day 7	day 14	day 28	day 7	day 14	day 28	day 7	day 14	day 28
14-Methylhexadecanoic acid	0.92	0.84	0.81	1.06	0.85	0.94	1.11	0.92	1.03
16-Methylheptadecanoic acid	0.68	0.56	0.49	0.88	0.80	0.96	0.98	1.01	1.07
17-Methyloctadecanoic acid	0.81	0.51	0.54	0.90	0.79	0.89	1.22	0.91	0.98
3-Hydroxybutyrate				1.24	1.18	0.97	0.95	1.02	0.91
3-O-Methylsphingosine [b]	0.81	0.69	0.80	0.88	0.68	0.72	1.00	0.86	0.93
Alanine	0.69	0.73	0.98	0.94	0.88		1.18	0.88	0.82
alpha-Tocopherol	0.72	0.78	0.82	0.96	0.80	0.86	1.09	0.97	0.96
Arachidonic acid (C20:cis[5,8,11,14]4)	0.75	0.70	0.72	1.02	0.86	0.97	0.96	0.96	1.03
Cholesterol	0.95	0.72	0.82	0.92	0.89	0.94	1.03	1.03	0.99
Coenzyme Q10	0.67	0.51	0.60	0.66	0.69	0.70	0.94	0.88	1.07
dihomo-gamma-Linolenic acid (C20:cis[8,11,14]3)	1.51			1.00	1.14	1.33	0.87	1.09	1.05
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)	0.88	0.71	0.56	1.08	0.90	0.83	1.14	1.05	1.01
Dodecanol	0.95	0.72	0.65	1.15	0.77	0.86	0.86	0.91	0.93
Elaidic acid (C18:trans[9]1)	0.93	0.76	0.77	1.01	0.96	0.98	0.91	0.88	0.99
Glycerol phosphate, lipid fraction	0.87	0.81	0.75	1.03	0.86	0.80	0.96	0.83	0.91
Heptadecanoic acid (C17:0)	0.78	0.62	0.76	0.86	0.82	0.85	0.97	0.85	1.13
Isoleucine	0.87	0.81	0.96	1.00	0.95	1.19	0.96	0.97	0.93
Leucine	0.84	0.86	1.02	0.96	0.92	1.22	0.95	0.97	0.88
Linoleic acid (C18:cis[9,12]2)	0.93	0.72	0.69	1.02	0.87	0.90	0.81	0.72	0.79
Linolenic acid (C18:cis[9,12,15]3)	0.93	0.68	0.57	1.19	1.05	1.18	0.71	0.70	0.91
Lysine	1.07			0.95	0.88	0.90	0.89	0.92	0.81
Lysophosphatidylcholine (C:17) [b]	0.70	0.67	0.73	0.80	0.88	0.92	0.96	0.89	0.88
Methionine	0.76	0.74	0.83	0.91	0.86	1.02	1.08	0.94	0.91
myo-Inositol, lipid fraction	0.83	0.82	0.82	0.93	0.86	0.93	0.90	0.97	0.84
Myristic acid (C14:0)	0.72	0.71	0.52	0.94	0.86	0.90	0.58	0.86	0.90
Pantothenic acid	1.19			1.19	1.15	1.21	1.17	1.08	0.97
Phosphatidylcholine (C16:0, C20:4) [b]	1.00			0.97	1.01	1.01	0.97	1.00	1.05
Phosphatidylcholine (C18:0, C22:6) [b]	0.90	0.89	0.79	0.94	0.92	0.88	0.95	0.96	0.94
Phosphatidylcholine No 02 [b]	0.81	0.73	0.71	1.03	1.05	0.96	1.05	0.89	0.87
Proline	0.79	0.83	0.86	0.93	0.95	1.02	0.94	0.93	0.92
Serine	0.75	0.86	0.89	0.90	1.01	1.07	0.86	0.98	0.88
Stearic acid (C18:0)	0.76	0.69	0.67	0.91	0.85	0.87	0.91	0.99	0.96
Threonine	0.75	0.76	0.82	0.92	0.99	0.93	0.97	0.89	0.90
trans-4-Hydroxyproline	0.84	0.88	0.87	1.07	0.91	0.90	0.95	0.99	0.90
Triacylglyceride (C18:1, C18:2) [b]	0.64	0.56	0.49	1.03	0.83	0.73	0.62	0.81	0.95
Unknown lipid (28000470)	0.78	0.63	0.58	0.93	0.85	0.66	0.94	0.85	0.76
Unknown lipid (28000473)	0.61	0.37	0.45	0.88	0.75	0.87	0.81	0.79	0.77
Unknown lipid (68000015)	0.88	0.78	0.66	0.94	0.82	0.75	1.02	1.03	0.91
Unknown lipid (68000017)	0.82	0.71	0.83	0.85	0.78	0.80	0.96	0.92	0.94
Unknown lipid (68000020)	0.76	0.57	0.61	0.89	0.80	0.77	0.93	0.80	0.95
Unknown lipid (68000028)	0.44	0.66	0.28	1.24	0.88	1.16	0.68	0.93	0.97
Unknown lipid (68000038)	0.61	0.67	0.81	0.94	0.90	1.00	1.13	0.96	1.04
Unknown lipid (68000047)	0.91	0.83	0.78	0.95	0.89	0.90	0.99	1.01	1.02
Unknown lipid (68000053)	0.87	0.92	0.89	0.96	0.99	0.92	0.96	1.09	1.03
Unknown lipid (69000059)	0.56	0.51	0.38	1.04	0.92	0.97	0.86	0.74	0.86
Unknown polar (58000136)			1.14	1.10	0.97	0.87	0.99	1.04	1.15
Valine	0.88	0.85	0.97	1.01	0.94		0.99	0.94	0.85

[a] Structure annotation is based on strong analytical evidence (e.g., combinations chromatography, mass spectrometry, chemical reactions, deuterium-labeling, database and literature search and comparison to similar/homologue/isomeric reference compounds).
[b] Metabolite exhibits identical qualitative analytical characteristics (chromatography and mass spectrometry) compared to a metabolite with footnote a. Further structural and analytical investigations of this metabolite are still pending.

and 7000 ppm DBP resulted in a pattern that was more profound than the individual compound patterns. This is also demonstrated by the fact that an additional 11 metabolites were found to be changed at a level of statistical significance of $p < 0.05$ (2 increased and 9 decreased). For most of the 11 additional metabolites a trend towards a change in a particular direction could already be seen for the individual compounds, and it would seem that the combination of the treatment resulted in a more pronounced and consistent expression of the metabolite changes, thus attaining statistical significance.

Table 4
Metabolite level changes in *female* Wistar rats treated with 7000, 1000 and 150 ppm DBP for 4 weeks. Metabolite levels were measured at study days 7, 14 and 28. Metabolites are mentioned with at least at two of three study days increased (red marked) or decreased levels (yellow marked; WELCH *t*-test, *p*-value ≤ 0.05) in one of the three mentioned dose groups. Figures represent relative changes of the median metabolite levels compared to controls (NA = not analysed).

Metabolite	DBP 7000 ppm			DBP 1000 ppm			DBP 150 ppm		
	day 7	day 14	day 28	day 7	day 14	day 28	day 7	day 14	day 28
3-Hydroxybutyrate				1.24			0.93	0.98	0.95
Alanine	0.75	0.82	1.00	0.75	0.79	1.07	0.83	0.84	1.17
Arginine	0.84	0.76	0.92	0.79	0.92	0.93	0.96	0.93	1.09
Cortisol	NA	0.26		1.42		0.95	0.94	0.05	2.29
Cysteine	0.87	0.89	1.00	0.85	0.83	0.95	0.96	1.01	0.98
Glutamate			0.88	0.98	1.18	1.03	1.15		
Glycine		1.04		1.02	1.01	1.09	1.09	1.05	1.14
Methionine	0.83	0.77	0.97	0.79	0.89	0.89	1.10	1.04	1.13
Pantothenic acid				1.03	1.06		1.07	1.25	
Phosphatidylcholine (C18:0, C18:2) [b]	0.97	1.00	0.96	0.98	0.96	1.00	1.00	1.02	1.00
Phosphatidylcholine (C18:2,C20:4) [b]			1.03	1.02		1.07	1.04	1.04	1.00
Tyrosine	0.80	0.75	0.99	0.96	0.86	1.02	1.12		1.31

[a] Structure annotation is based on strong analytical evidence (e.g., combinations chromatography, mass spectrometry, chemical reactions, deuterium-labeling, database and literature search and comparison to similar/homologue/isomeric reference compounds).

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Table 5b
Metabolite level changes in *female* Wistar rats treated with 3000 ppm DEHP for 4 weeks. Metabolite levels were measured at study days 7, 14 and 28. Metabolites are mentioned with at least at two of three study days increased (red marked) or decreased levels (yellow marked; WELCH *t*-test, $p \leq 0.05$) in the mentioned dose group. Figures represent relative changes of the median metabolite levels compared to controls.

Metabolite	DEHP 3000 ppm		
	day 7	day 14	day 28
3-Hydroxybutyrate			
3-O-Methylsphingosine [1]		1.74	
5-O-Methylsphingosine [1]			
Arginine	0.73	0.84	0.87
Coenzyme Q10	1.11		
Coenzyme Q9			
Lignoceric acid (C24:0)			
Normetanephrine	0.93	0.82	0.69
Palmitic acid (C16:0)			1.27
Pantothenic acid		1.50	
Sphingomyelin (d18:1, C16:0) [1]		1.37	
Sphingomyelin No 02 [2]			
Triacylglyceride (C18:1, C18:2) [1]	0.74		
Tyrosine	0.86	0.81	0.92
Unknown lipid (68000021)			1.41
Unknown lipid (68000033)	0.88	0.81	0.91
Unknown lipid (68000038)	0.79	0.83	0.81
Unknown lipid (68000060)			

[a] Structure annotation is based on strong analytical evidence (e.g., combinations chromatography, mass spectrometry, chemical reactions, deuterium-labeling, database and literature search and comparison to similar/homologue/isomeric reference compounds).
[b] Metabolite exhibits identical qualitative analytical characteristics (chromatography and mass spectrometry) compared to a metabolite with footnote a. Further structural and analytical investigations of this metabolite are still pending.

Dose–response relationship for metabolite patterns in the combined groups (Tables 8 and 9). The combined administration of 3000 ppm DEHP and 100 ppm DBP did not result in a biologically relevant difference in the metabolite profile in males or females. In males, one single metabolite (3-hydroxybutyrate) attained consistent statistical significance ($p < 0.05$, at least two points in time) in the (low dose DBP) combination group relative to the DEHP only treatment group. However, it can be seen that this metabolite in the DEHP only group was also numerically increased, and that the extent of this increase was very similar to that of the combination group. In contrast, a few metabolites did not attain statistical significance ($p < 0.05$) in the combination group. For these observations too, it has to be noted that the numerical value was similar in the DEHP only and the low dose DBP combination group.

For females, glycochenodeoxycholic acid was consistently decreased ($p < 0.05$) in the low dose combination group, whereas the DEHP group alone did not show such a consistent effect. Threonine and phosphatidylcholines no. 1 and no. 4 were consistently decreased in the low dose combination group. As these metabolites showed a similar trend in the DEHP only group, these are not considered to be indicative of a specific DBP contribution.

Table 6
Heat-map of metabolite level changes in *male* Wistar rats treated with 7000 ppm DBP, 3000 ppm DEHP or a combination of both compounds for 4 weeks. Metabolite levels were measured at study days 7, 14 and 28. Metabolites are included when at least at two of three study days increased (red marked) or decreased levels (yellow marked; WELCH *t*-test, $p \leq 0.05$) in one of the three mentioned dose groups were noted.

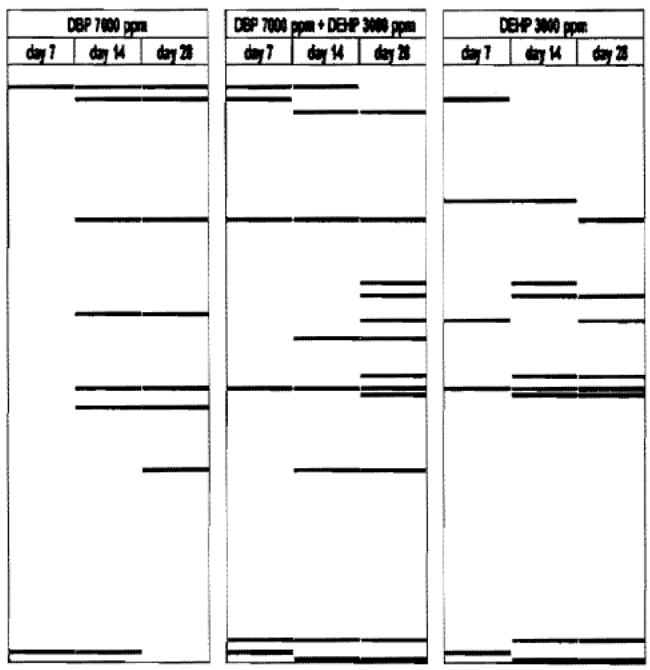
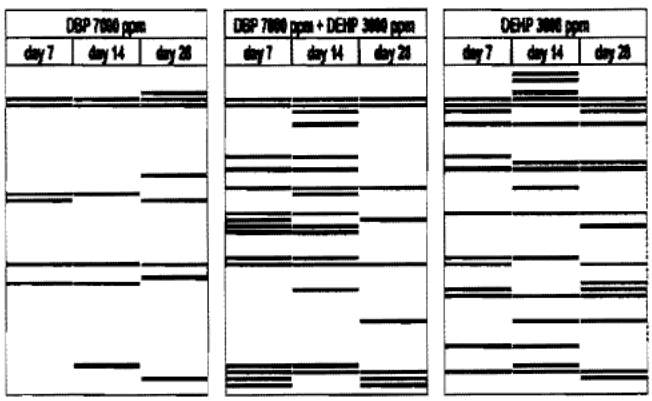


Table 7
Heat-map of metabolite level changes in *female* Wistar rats treated with 7000 ppm DBP, 3000 ppm DEHP or a combination of both compounds for 4 weeks. Metabolite levels were measured at study days 7, 14 and 28. Metabolites are included when at least at two of three study days increased (red marked) or decreased levels (yellow marked; WELCH *t*-test, $p \leq 0.05$) in one of the three mentioned dose groups were noted.



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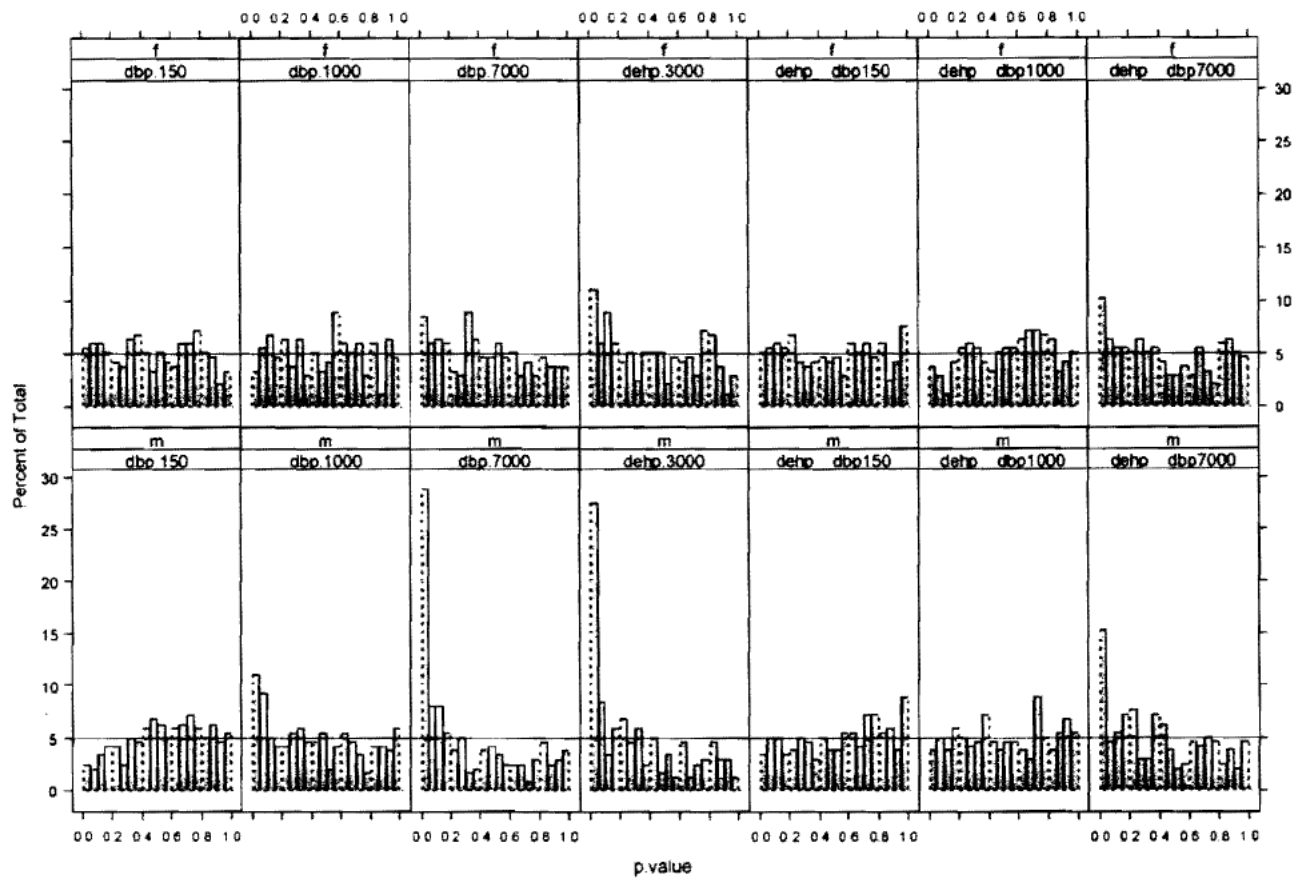

Fig. 2. Distribution of unadjusted *p*-values of seven phthalate contrasts stratified by sex (top row: female; botton row: male). Bar width is 0.05, so each bar represents the number of metabolites in the particular 5% *p*-value range. Treatment contrasts refer to day 28, the time point revealing the strongest effects in terms of the number of significant metabolites. Each panel represents the *p*-value distribution of 238 metabolites. Main effects and interactions are clearly observed for single ("dbp.7000", "dehp.3000") and combined ("dehp.dbp.7000") high dose treatments.

significant interaction effects (clearly more than 5% of metabolite *p*-values are below 0.05) can be discerned for both genders exclusively affecting the combination of both high dose groups ("DEHP_DBP 7000": 3000 ppm DEHP + 7000 ppm DBP), see Fig. 2. Some insight into the interaction structure can be obtained by omitting those metabolites significant ($p \leq 0.05$) under 3000 ppm DEHP and 7000 ppm DBP treatment, see Fig. 3. After omission of these metabolites no more interaction effects are found significant (effects at $p \leq 0.05$ at or below 5% significance threshold for "DEHP_DBP 7000"). This observation gives already evidence against over-additive in favor of under-additive effects, because over-additivity would suggest significant combined effects from non-significant main effects. The latter, however, is not observed in Fig. 3.

Further understanding of the effect structure was obtained by directly investigating the effect size of the different treatment regimes. Fig. 4 shows a scatter plot of the estimated combined effects versus the sum of the estimated single dose effects. Additivity of effects is indicated by the diagonal (dashed line) in Fig. 4. Thus, the experimental results indicate underadditive effects as revealed by an approximate logistic relationship (highlighted by the black line in Fig. 4) with marked ceiling effects at the tails of the distribution. Ceiling effects are more pronounced in the male compared to the female group, which is a direct consequence of the small regulation strength in the female group. This again demonstrates the different sensitivity of male and female animals under phthalate exposure.

4. Discussion

We have used the term metabolomics for a whole animal system based approach using LC–MS/MS and GC–MS as the analytical method in order to distinguish this technique from the traditional NMR based approaches (metabonomics). With the available analytical procedures and techniques, 238 unique blood metabolites with a molecular weight from about 80 to 1000 Da could be reliably detected and quantified. As male and female rats generally demonstrate different metabolic profiles to the same treatment (van Ravenzwaay et al., 2007) we have analysed the metabolomics data for males and females separately. Comparing the metabolites altered at 7000 ppm DB P in males and females it can be noted that only 4 metabolites (pathothenic acid, 3-hydroxybutyrate, alanine and methionine) were regulated in the same manner. Gender-specific metabolome differences in mice, rats and humans have also been reported by other groups (Plumb et al., 2005; Kochhar et al., 2006, Strauss et al., 2009).

In males treated with DBP there was clear response at 7000 ppm with 17% of all investigated metabolites being regulated. There was only a very weak response at 1000 and no consistent response at 150 ppm. In females at 1000 ppm the response was marginal (6 metabolites at 1 or two time points) and at 150 ppm even less changes were noted. According to the criteria proposed by ECETOC (2007) to define no effect levels in omics studies ("only specific patterns of change should be used to conclude that a potentially relevant biological effect is taking place") the lack of any pattern in the

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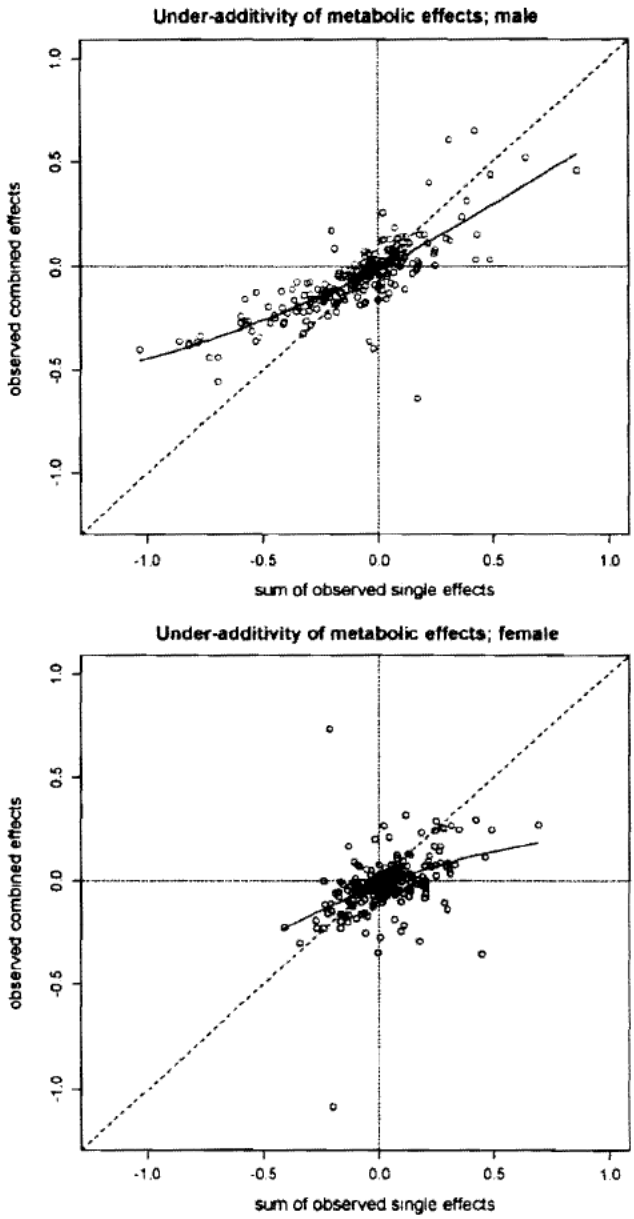

Fig. 4. Scatter plot of the observed combined high dose effects (vertical axis) versus the sum of the corresponding observed single-treatment high dose effects (horizontal axis) for males (top) and females (bottom). The dashed line marks the diagonale ("additivity of effects") and the solid line is a logistic fit to the data indicating under-additivity.

the combined profile was more pronounced than the individual profiles. In males the interaction was less pronounced. Upon quantitative analysis for both sexes, it was noted that the combined effects were sub-additive, i.e. $1 + 1 < 2$, an effect commonly known in toxicology as ceiling effect. A possible explanation for this observation may be related to the fact that the most pronounced effect observed in the present studies are found in the liver and in particular lipid metabolism, which is likely linked to the fact that both compounds are strong inducers of peroxisome proliferation. We speculate that the less than linear additive effects at the combined high dose level are related to the fact that a near maximum response has been obtained. The metabolome analysis in this context is similar to the P-CoA) oxidation activity which also increases less than linear. Moreover, as the studies were performed in adult animals, the sensitivity of such animals with respect to effects on the testes is acknowledged to be less than in neo-nates or pups.

From a risk management point of view, the main question is whether the exposure to mixtures of chemicals at low, realistic doses is of real health concern. There is not much information on prolonged, repeated toxicity studies on combinations of chemicals at low (nontoxic) or subtoxic doses (Krisshnan and Brodeur, 1991; Heindel et al., 1994). In acute and subacute toxicity studies in rats it has been shown that combined oral administration of compounds at the "no-observed-adverse-effect level" (NOAEL) of each of them did not lead to clear additivity or synergism of effects, provided the mechanism of action of the compounds was dissimilar (Jonker et al., 1993). In a 4-week toxicity study with mixtures consisting of four nephrotoxicants with similar mode of action, it was shown that the dose-additivity rule could be applied (Feron et al., 1995b) at dose levels around the NOAEL. These 4-week toxicity studies were interpreted based on the common approaches in the assessment of mixtures; for a combination of compounds with a similar mode of action, one might expect dose-addition (additivity), whereas compounds with a dissimilar mode of action may show less than additivity (Plackett and Hewlett, 1952; Mumtaz et al., 1994). The results of our studies demonstrate, that even in the case that two compounds have a similar mode of action, not always, additivity is noticed. This would mean, that applying the concept of additivity for compounds with similar modes of action can be considered to be a conservative, and thus reasonable, first approach for risk assessment.

Of equal importance is the question concerning interaction at the level of the NOEL or at the lower end of the dose–response curve. In an extensive 4-week oral/inhalatory study in male Wistar rats, published by Groten et al., 1997, the toxicity (clinical chemistry, hematology, biochemistry and pathology) of combinations of nine compounds was examined. The study comprised 20 groups, 4 groups in the main part ($n = 8$) and 16 groups in the satellite part ($n = 5$). In the main study, the rats were simultaneously exposed to mixtures of all nine chemicals (dichloromethane, formaldehyde, aspirin, di(2-ethylhexyl) phthalate, cadmium chloride, stannous chloride, butyl hydroxyanisol, loperamide, and spermine) at concentrations equal to the "lowest-observed-adverse-effect level" (LOAEL), NOAEL, or 1/3 NOAEL. The authors concluded that simultaneous exposure to these nine chemicals does not constitute an evidently increased hazard compared to exposure to each of the chemicals separately, provided the exposure level of each chemical in the mixture is at most similar to or lower than its own NOAEL. The results are in line with a large experiment, reported by Ito et al. (1995) in which the absence of interaction was demonstrated for the ingestion of 20 pesticides at their respective acceptable daily intake levels in an oral carcinogenicity study in rats.

In conclusion, the results of our study indicate that a DBP dose level causing no metabolomic changes by itself, also had no effect on the metabolome of a high dose of DEHP. Interaction of compounds was seen at high dose levels of DBP and DEHP, but the combined effects were less than additive was less than linear. These results and future work may help to set rules for the risk assessment of mixitures.

Conflict of interest

BASF is a producer of phthalates.

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EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

5th Working group meeting on Toxicity of Chemical Mixtures (TCM)

Meeting date: 24 January 2011 starting at 10:00

B232 room 02/17A – Brussels

Minutes

1. WELCOME AND APOLOGIES

The chairman welcomed the participants and participants and indicated the apologies.

2. ADOPTION OF THE AGENDA

The agenda was adopted.

3. APPROVAL OF THE MINUTES OF THE LAST MEETING.

The minutes were approved.

4. DECLARATION OF INTEREST ON MATTERS ON THE AGENDA

There were no interests declared.

5. DISCUSSION OF THE OPINION AND DISTRIBUTION OF TASKS

- There is a need for this opinion to offer a framework for the assessment of mixtures.

- There are some inconsistencies which need to be clarified.

- To clarify our position vis-à-vis The State of the Art report.

- Definitions of simple and complex mixtures are still missing.

- To distinguish between the levels of risk assessment (EU, national, local).

- There should be a contribution or at least comment on the mixtures in cosmetics and pharmaceuticals by an external expert.

- To provide examples and also to refer to AB's manuscript (circulated earlier) in the text (page 9).

- A decision tree (tiered approach) to be developed to help regulators decide when adverse effects might be expected.

- To link the text on page 11 with the previous section.

- To send some examples of long-term testing (biocides directive).

- A sentence to be added about the cosmetics regulation.

- To write some text about the toxi... approach and a footnote about the environmental ... (the bees).

- The limitations and advantages for each of the approaches are needed.

- To check line 26 (page 16) and to add a text for the effect on the environment of chemicals of low-dose concentrations.

- The epidemiological evidence and the text on uncertainty are to be modified and inserted in the opinion.

- The current risk assessment methodology for evaluation of single chemicals may be used; however, all possible sources of exposure are to be considered.

- The answers of question 1 to be categorized according to the three modes of action.

- In the answers of question 1, it should be clearly indicated that this WG does not agree with the evidence presented in The State of the Art report about the effects of low-dose concentrations because this is the key issue and the focus of attention by the interest groups (NGOs and industry).

- In the answers of question 4 it should be indicated that the margin-of-exposure (MoE) approach is the best for no-known-threshold-effect substances.

- In question 5 – there are no major knowledge gaps in terms of methodology. There is a data gap, i.e. information needed to be able to apply the already developed approaches for risk assessment.

- In question 6 – research is needed to identify a different approach to evaluating large amounts of data.

- The EPA flow chart and the IPCS document may serve as a starting point for the development of better criteria with priorities for risk assessment of mixtures.

6. NEXT MEETING – 29 MARCH 2011

7. ANY OTHER BUSINESS

There was none.

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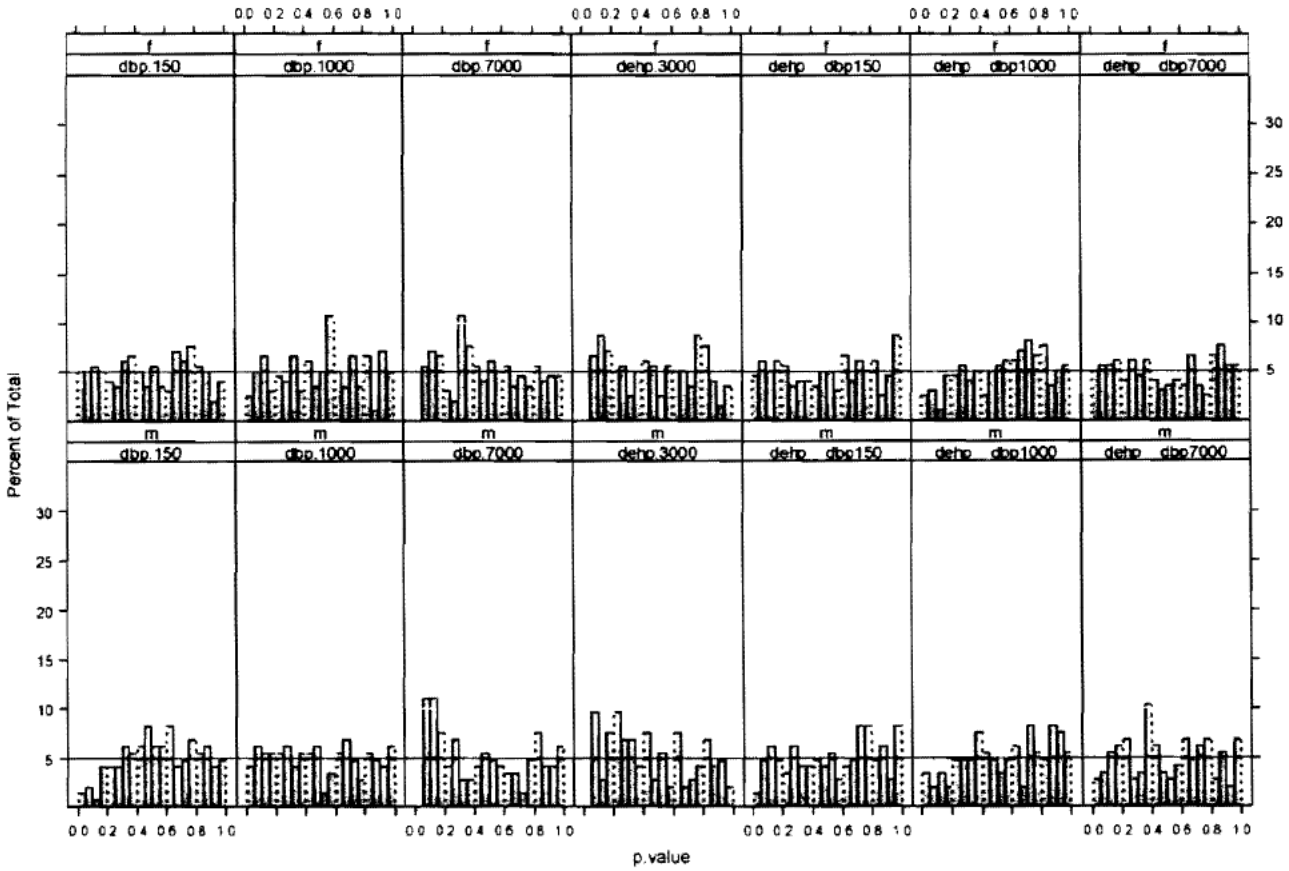

Fig. 3. Percentages of unadjusted *p*-values of the contrasts from Fig. 2 after omitting "dbp.7000"- and "dehp.3000"-significant metabolites. The number of significant metabolites in combined high-dose treatment is reduced to the 5% false positive level (red line, "dehp_dbp.7000"). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

150 ppm group indicates that this dose represents a metabolomic no observed effect level. In addition, the number of metabolite changes is less than can be expected from pure change (if *p* is set at ≤0.05, then up to 11 metabolites (out of 238) could be expected to be changed by chance).

Based on a series of reference compounds, typical metabolite changes (patterns) have been established for different toxicological modes of action using metabolomics techniques (van Ravenzwaay et al., 2010). For three different modes of action involving the liver, metabolite patterns were published. The pattern of one of these modes of action, peroxisome proliferation, matches that of DBP and DEHP quite well. Metabolite changes typical for peroxisome proliferators include reduced values for coenzyme q10, 16- and 17-methylheptadecanoic acid, threonine, proline and trans-4hydroxyproline, as well as increased values for eicosatrienoic acid. Compounds inducing peroxisome proliferation, enhance enzyme activity which catabolizes fatty acids and contribute essentially to the β-oxidation of long chain fatty acids. Thus, from a biochemical perspective at least some of the metabolite changes observed following the treatment with these peroxisome proliferators can be explained; e.g. reduced concentrations of several long chain fatty acids such as 14-methylhexadecanoic acid, 16-methyl-heptadecanoic acid, and linoleic acid (C18:cis[9,12, 16]3). The concomitant increase of eicosatrienoic acid can be seen as the result of this long-chian fatty acid conversion and has been described by Kawashima et al., 1990. The observed reduction in threonine, proline and trans-4-hydroxyproline remains to be elucidated. It should be noted that particularly the changes in the lipids were more pronounced in males than in females. The increased cyanide-insensitive Palmitoyl-Co-A oxidation can be regarded as a confirmation of the peroxisome proliferating potential of both compounds. Moreover, the fact that the increase in this enzyme activity was about twice as high in males as in females suggested that the enhanced lipid metabolite changes in this sex are indeed mechanistically related to peroxisome proliferation.

4.1. Combination toxicology

One of the purposes of our investigations was to study the interaction of two chemicals simultaneously applied to rats at the level of metabolite profiles. This approach for assessing the combined toxicity of defined chemical mixtures has been applied by several research groups for e.g. nephrotoxicants, pesticides, carcinogens, and/or fertilizers (Jonker et al., 1993; Charturved, 1993; Heindel et al., 1994; Feron et al., 1995a; Ito et al., 1995). The results of our studies indicate that the co-administration of 150 ppm DBP (a toxicological and metabolomics NOEL) in rats receiving a high dose of DEHP (3000 ppm) does not have any effect on the metabolite profile (i.e. in simple terms: 1 + 0 = 1). The co-administration of 1000 ppm DBP (which induces by itself only a moderate metabolite profile in both males and females) has a marginal effect on the high dose DEHP profile. No additional metabolite level changes were noted, but some of the metabolites where regulated at a numerically higher level – resulting in a dominant DEHP profile. It was only when both high dose levels were combined (DBP 7000 ppm + DEHP 3000 ppm) that a clear interaction of both compounds on the metabolite profile was seen. Particularly in females

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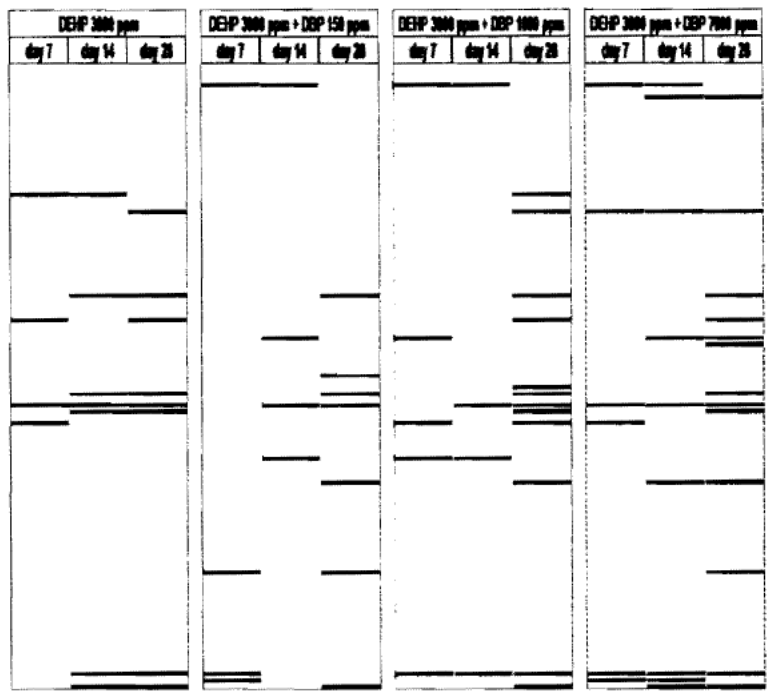
Table 8
Haet-map of metabolite level changes in *male* Wistar rats treated with 3000 ppm DEHP only as well as with 3000 ppm DEHP combined with 150, 1000 and 7000 ppm DBP for 4 weeks. Metabolite levels were measured at study days 7, 14 and 28. Metabolites are included when at least at two of three study days increased (red marked) or decreased levels (yellow marked; WELCH *t*-test, $p \leq 0.05$) in one of the four mentioned dose groups were noted.

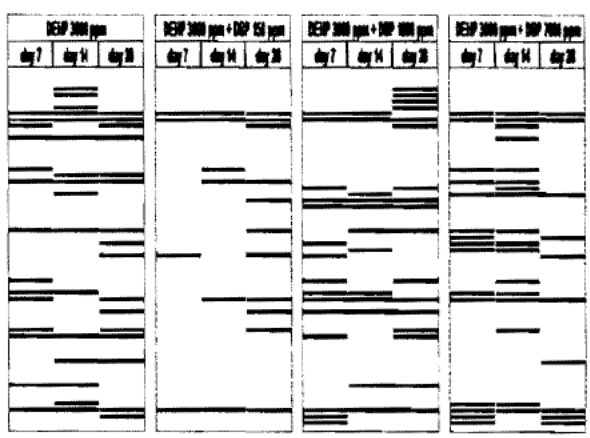


The combined treatment of 3000 ppm DEHP and 1000 ppm DBP in males resulted in a few more metabolites being consistently changed (statistically significantly at $p < 0.05$ for at least two points in time) in the combination group compared to the DEHP only group. However, all of these metabolites were changed in a similar manner with DEHP alone, however only a single point in time.

For females, a few metabolites were novel, compared to the DEHP alone treatment group. These consisted of alanine, methionine (reduced), and glycine (increased). As these metabolites were found to be changed in a similar manner by DBP alone, it seems likely that the changes in these metabolites in the combination group can be attributed to the effects of DBP.

It should also be noted that some metabolites, which were found regulated with 3000 ppm DEHP alone did not attain statistical significance in the combination treatment group. Whether this change has a true biological background, or if it is related to chance, cannot be determined with certainty, as the biological significance of these metabolites is not yet known.

Table 9
Heatmap of metabolite level changes in *female* Wistar rats treated with 3000 ppm DEHP only as well as with 3000 ppm DEHP combined with 150, 1000 and 7000 ppm DBP for 4 weeks. Metabolite levels were measured at study days 7, 14 and 28. Metabolites are included when at least at two of three study days increased (red marked) or decreased levels (yellow marked; WELCH *t*-test, $p \leq 0.05$) in one of the four mentioned dose groups were noted.



3.4. Statistical analysis: linear mixed-effects model

The factorial nature of the design was exploited by setting up mixed-effects models for all metabolites individually and by analysing the main and interaction contrasts as defined in Section 2.

Evaluation of the test statistics of the sex-stratified models of the seven treatment contrasts revealed an increase of the effect size in time with day 28 showing the most pronounced effects on the metabolome (details not shown here). Therefore, treatment contrasts were defined with reference to day 28.

Fig. 2 shows an overview of the test statistics of the phthalate contrasts at day 28 stratified by sex. In the 150 ppm DBP low dose group no treatment effects were found significant at the 5% test level (effects with *p*-value ≤ 0.05 are below or at the 5% threshold marked by a red line). However, significant high dose effects (3000 ppm DEHP and 7000 ppm DBP respectively) are clearly detectable in Fig. 2 with approximately 30% of the metabolites found significant in the male and 10% in the female groups ($p \leq 0.05$). So, the strength of the phthalates with respect to the metabolite profile in the female group is approximately one third of that in the male group. In addition to the above main effects

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Table 5a
Metabolite level changes in *male* Wistar rats treated with 3000 ppm DEHP for 4 weeks. Metabolite levels were measured at study days 7, 14 and 28. Metabolites are mentioned with at least at two of three study days increased (red marked) or decreased levels (yellow marked; WELCH t-test, $p \leq 0.05$) in the mentioned dose group. Figures represent relative changes of the median metabolite levels compared to controls.

	DEHP 3000 ppm		
Metabolite	**day 7**	**day 14**	**day 28**
14-Methylhexadecanoic acid	0.75	0.69	0.73
16-Methylheptadecanoic acid	0.48	0.41	0.41
17-Methyloctadecanoic acid	0.44	0.35	0.45
3-O-Methylsphingosine [1)]	0.82	0.67	0.97
Arachidonic acid (C20:cis[5,8,11,14]4)	0.67	0.61	0.82
Behenic acid (C22:0)	0.74	0.74	0.82
Cholesterol	0.77	0.78	0.82
Choline plasmalogen (C18, C20:4) [1)]	0.87	0.82	0.98
Coenzyme Q10	0.60	0.50	0.55
Creatine			1.54
Diacylglyceride (C18:1, C18:2) [2)]	0.71	0.52	0.69
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)	0.55	0.48	0.53
Dodecanol	0.77	0.70	0.80
Eicosanoic acid (C20:0)	0.65	0.63	0.70
Elaidic acid (C18:trans[9]1)	0.80	0.63	0.80
Galactose, lipid fraction	0.72	0.77	0.75
Glycerol, lipid fraction	0.50	0.49	0.58
Heptadecanoic acid (C17:0)	0.69	0.61	0.66
Leucine	1.04		
Linoleic acid (C18:cis[9,12]2)	0.62	0.47	0.58
Linolenic acid (C18:cis[9,1...	0.54	0.44	0.47
Lysophosphatidylcholine (C16:0) [2)]		1.15	
Lysophosphatidylcholine (C17:0) [2)]	0.67	0.75	0.66
Lysophosphatidylcholine (C18:0) [1)]	0.87	0.95	0.93
myo-Inositol, lipid fraction	0.75	0.71	0.79
myo-Inositol-2-phosphate, lipid fraction	0.67	0.52	0.89
Myristic acid (C14:0)	0.45	0.47	0.56
Nervonic acid (C24:cis[15]1)	0.55	0.64	0.71
Ornithine	1.10		
Palmitoleic acid (C16:cis[9]1)	0.58	0.45	0.69
Pantothenic acid			
Phenylalanine	1.04		
Phosphate, lipid fraction	0.88	0.73	1.06
Phosphatidylcholine (C16:1, C18:2) [1)]	0.60	0.53	0.54
Phosphatidylcholine (C18:0, C18:1) [1)]	0.79	0.81	0.86
Phosphatidylcholine (C18:0, C22:6) [1)]	0.73	0.70	0.69
Phosphatidylcholine (C18:2, C20:4) [1)]	0.87	0.87	0.88
Phosphatidylcholine No 02 [1)]	0.69	0.69	0.63
Proline	0.84	0.90	0.90
Serotonin (5-HT)	0.25	0.77	0.12
Stearic acid (C18:0)	0.63	0.60	0.67
Triacylglyceride (C16:0, C16:1) [1)]	0.51	0.58	0.71
Triacylglyceride (C16:0, C18:2) [1)]	0.81	0.56	0.62
Triacylglyceride (C18:1, C18:2) [1)]	0.44	0.41	0.43
Triacylglyceride (C18:2, C18:2) [1)]	0.61	0.42	0.49
Triacylglyceride (C18:2, C18:3) [1)]	0.32	0.27	0.35
Unknown lipid (28000470)	0.74	0.52	0.55
Unknown lipid (28000473)	0.42	0.36	0.33
Unknown lipid (28000493)	0.59	0.48	0.69
Unknown lipid (68000009)	0.70	0.70	0.86
Unknown lipid (68000015)	0.81	0.79	0.79
Unknown lipid (68000017)	0.66	0.61	0.73
Unknown lipid (68000020)	0.54	0.49	0.64
Unknown lipid (68000028)	0.31	0.35	0.20
Unknown lipid (68000033)	0.82	0.77	0.79
Unknown lipid (68000034)	0.43	0.53	0.49
Unknown lipid (68000038)	0.55	0.52	0.55
Unknown lipid (68000044)	0.50	0.42	0.41
Unknown lipid (68000053)	0.81	0.84	0.88
Unknown lipid (68000056)	0.48	0.26	0.34
Unknown lipid (68000057)	0.81	0.54	0.71
Unknown lipid (68000058)	0.47	0.51	0.48
Unknown lipid (68000059)	0.36	0.29	0.34
Unknown lipid (68000060)	1.19		
Valine	1.07		

[a] Structure annotation is based on strong analytical evidence (e.g., combinations chromatography, mass spectrometry, chemical reactions, deuterium-labeling, database and literature search and comparison to similar/homologue/isomeric reference compounds).
[b] Metabolite exhibits identical qualitative analytical characteristics (chromatography and mass spectrometry) compared to a metabolite with footnote a. Further structural and analytical investigations of this metabolite are still pending.

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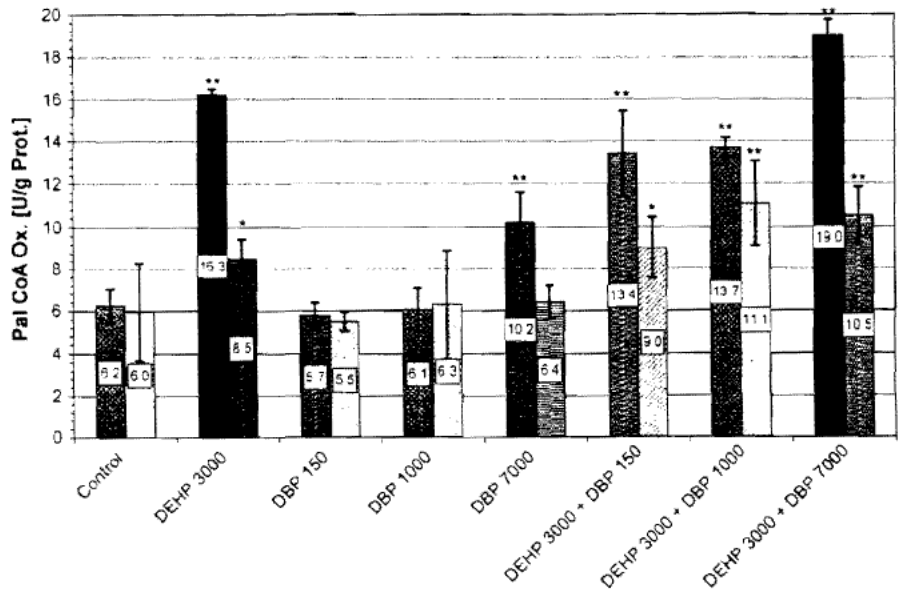

Fig. 1. Means ± standard deviation of the cyanide-insensitive Palmitoyl CoA oxidation per g liver protein measured in the controls and 7 dose groups (5 rats per dose group and sex and 10 rats per sex in the controls). The values of the dose groups were compared to the controls with the two-sided WILCOXON test (*$p < 0.05$; **$p < 0.01$).

pared to untreated controls, the level of statistical significance increased from $p < 0.05$ in the 3000 ppm DEHP group, to $p < 0.01$ in the 3000 ppm DEHP + 1000 ppm DBP group (see Fig. 1). In males receiving 3000 ppm DEHP + 7000 ppm DBP, P-CoA oxidation was higher than in all other treatment groups. In females receiving the high dose combination treatment P-CoA activity was similar to the combined treatment of DEHP and 1000 ppm DBP.

3.2. Metabolite profiles

3.2.1. DBP

At 7000 ppm, a total of 47 metabolite levels, out of 238, were changed in *male* rats following DBP treatment for 28 days. Most of these were decreased (41) and only 6 were increased. The profile (i.e., number and size of metabolite level changes) was more pronounced at day 14 and 28 compared to day 7 (Table 3).

At 1000 ppm only 3 metabolites were consistently changed (statistical significance achieved on at least 2 study days), all of them showing a reduction. For 8 metabolites a decrease (i.e., metabolite levels in dosed rats decreased compared to controls) was observed on a single occasion. All of these metabolites were also decreased at the high dose level. There were two cases (both up regulated) in which metabolites at 1000 ppm showed a response which was dissimilar to the high dose level. At 150 ppm there was 1 metabolite level consistently decreased, and 4 more metabolites (also decreased) on a single time point.

In *female* rats dosed with 7000 ppm DBP only 12 (out of 238) metabolites were consistently changed, 6 of these were increased, 6 decreased (Table 4). At 1000 ppm only 1 metabolite was changed at two time points and 5 metabolites were altered in a similar way as in the high dose group at one time point. At 150 ppm there were 2 metabolites (alanine, glutamate) consistently changed at two time points and two more (panthotenic acid, tyrosine) at one time point. One metabolite (glutamate) was increased in the high dose group and the low dose group, but not in the mid dose group.

3.2.2. DEHP

At 3000 ppm, a total of 65 metabolite levels, out of 238, were changed in *male* rats following DEHP treatment for 28 days, only 8 being increased and the majority (57) decreased. The profile appeared to be equally strong at all three time points. At 3000 ppm, a total of 18 metabolites, out of 238, were changed in *female* rats following DEHP treatment for 28 days, 13 being increased and 5 decreased (Tables 5a and 5b).

3.3. Combination DEHP and DBP

Comparing the metabolite profile of the males of the 3000 ppm DEHP and 7000 ppm DBP group, it can be seen (Table 6) that many metabolites were changed in a similar way for both compounds. Based on a level of statistical significance of $p < 0.05$ there were 19 metabolites which were only changed for DEHP (5 increased and 14 decreased), whereas there were only 8 metabolites regulated only for the DBP treated males (3 increased and 5 decreased). The combination of 3000 ppm DEHP and 7000 ppm DBP resulted in a pattern that was very similar to a combination of the individual patterns. Only 3 additional metabolites were found to be changed at a level of statistical significance of $p < 0.05$ (increased: 4-hydroxyphenyl-pyruvate, decreased: adrenaline, lysophosphatidylcholine (C18:2)). Taking into account the trend towards an increase of hydroxyphenyl-pyruvate in both the single DBP and DEHP group, this metabolite is most likely not new in terms of biochemical pathways, but rather enhanced in the level (consistency) of regulation and thus attaining statistical significance. Some of the metabolite changes (reduced values for coenzyme q10, 16- and 17-methylheptadecanoic acid, threonine, proline and trans-4hydroxyproline, as well as increased values for eicosatrienoic acid) which were observed for both compounds are typical metabolite changes observed with compounds known to be inducers of peroxisome proliferation.

A comparison of the metabolite profile of the female rats of the 3000 ppm DEHP and 7000 ppm DBP group, shows that some similarities between the metabolite profile of both treatment groups, but far less similarity than with male rats. The DEHP profile is more prominent than the one of DBP (Table 7). Based on a level of statistical significance at $p < 0.05$ there were 17 metabolites specifically (only occurring for one of the two compounds) regulated for DEHP (13 increased and 4 decreased), whereas there were 9 metabolites in DBP treated males specifically regulated (3 increased and 6 decreased). The combination of 3000 ppm DEHP

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posing a severe multiple testing problem. In metabolite profiling, however the sample size is usually much larger relative to the number of features (number of metabolites) thereby alleviating the multiple testing problem and facilitating the use of more advanced statistical modeling techniques such as ANOVA mixed-effects models.

Phthalate esters are used as plasticizers to impart flexibility and resilience to plastic products. The phthalate esters are not covalently bound in the products, but can migrate into the surrounding environment. Because the phthalate esters are used in such a wide variety of consumer products, human exposure to the phthalate esters is widespread. The phthalate diesters are rapidly converted to the monoesters in the rat and in the human (Albro et al., 1982; Anderson et al., 2001).

In rat dosed with dibutylphthalate (DBP) liver changes (hepatomegaly with peroxisome proliferation cytoplasmatic alterations, consistent with glycogen depletion as well as cholestasis) were the most prominent findings (Jansen, 1993; Schilling, 1992; NTP, 1995a,b). In males dosed with DBP the spermatogenesis was affected as a consequence of the degeneration of the germinal epithelium and a severe atrophy of the seminiferous tubules. In special studies examining testicular effects in rats, reduced serum testosterone, but increases in testicular testosterone levels as well as low testicular zinc concentrations as a consequence of an increased urinary zinc excretion were observed (Gray et al., 1982, 1983; Oishi and Hiraga, 1980; Srivastava et al., 1990).

Subchronic feeding studies in rats with DEHP revealed the same main target organs and similar findings as in the repeated dose studies with DBP (David et al., 1999): hepatomegaly with peroxisome proliferation as well as spongiosis hepatis was observed in the liver, as well as a bilateral aspermatogenesis with moderate seminiferous tubule atrophy and mild vacuolization in the Sertoli cells.

From the studies conducted it can be concluded that the toxicological profiles of DEHP and DBP are similar, but not identical. As human exposure to both compounds at the same time can occur, it is of importance to know the toxicological consequences of a combined exposure. In addition, the combined exposure to both compounds at dose levels resulting in toxicological effects, which are most likely beyond any human exposure, can be used to study the nature of the interactions of the compounds, i.e. to test if the combination of effects results in additivity (1 + 1 = 2), sub-additivity (1 + 1 < 2), or supra-additivity (1 + 1 > 2). The aim of this study thus was (1) to elucidate the individual metabolic profile of DBP and DEHP, (2) to determine the dose–response relationship of the metabolic profile of DBP and (3) to investigate the nature of the interaction of combined exposure to DEHP and DBP on the metabolic profile in a dose related manner.

2. Materials and methods

2.1. Animals and maintenance conditions

Wistar rats (Crl:WI(Han)) were supplied by Charles River, Germany at an age of 61–64 days and underwent an acclimatization period of 1 week. Male and female Wistar rats were randomized according to body weight and allocated to the dose groups before the beginning of the administration period. The animals were housed individually in standard cages (floor area 800 cm^2), supplied by Becker & Co., Castrop-Rauxel, Germany. Waste trays were fixed underneath the cages, containing bedding material (type 3/4 dust free embedding, supplied by SSNIFF, Soest, Germany). The animals were maintained in an air-conditioned room at a temperature of 20–24 °C, a relative humidity of 30–70%, and a 12 h light/12 h dark cycle. Before the animals' arrival, the room was completely disinfected using a disinfector ("AUTEX", fully automatic, formalin-ammonia-based terminal disinfector, supplied by Dr. Gruß KG, Neuss, Germany). During the study, the floor and walls were cleaned weekly with a solution of 0.1% Incidin® (supplied by Henkel, Düsseldorf, Germany) in water. Ground Kliba mouse/rat maintenance diet was supplied by Provimi Kliba SA, Kaiseraugst, Switzerland. The diet and drinking water were available *ad libitum* (except immediately before sampling) and regularly assayed for chemical contaminants and the presence of microorganisms.

2.2. Treatment of animals with compounds

The study was performed according to the German Animal Welfare legislation. The laboratory is AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) certified.

For each dose group, five rats per sex were either fed with a diet containing the individual test substance or a combination thereof. The doses were chosen based on the symptoms and no observed effect levels as described in literature (see Sections 1 and 4).

For each concentration, the test substances were weighed out and mixed with a small amount of food. Then corresponding amounts of food, depending on dose group, were added to this premix in order to obtain the desired concentrations. Mixing was carried out for about 10 min in a laboratory mixer. The dosed rats were compared to untreated maintenance diet controls. The individual and combinations doses selected for the present study, as well as the rational and purpose of investigation are shown in Table 1.

2.3. Blood sampling

Between 7:30 and 10:30 h, blood samples were taken from the retro-orbital sinus in all rats under isoflurane anesthesia (1.0 ml K-EDTA blood on study days 7, 14 and 28) after a fasting period of 16–20 h. The blood samples were centrifuged (10 °C, 2000 × g, 10 min) and the EDTA plasma was separated. The EDTA plasma samples were covered with nitrogen and frozen at −80 °C until metabolite profiling was performed.

2.4. Metabolite profiling

For mass spectrometry-based metabolite profiling analysis, K-EDTA plasma samples taken on study days 7, 14 and 28 were extracted by a proprietary method. Three types of mass spectrometry analysis were applied to all samples: GC–MS (gas chromatography–mass spectrometry) and LC–MS/MS (liquid chromatography–MS/MS) were used for broad profiling, as described in van Ravenzwaay et al. (2007). SPE-LC–MS/MS (solid phase extraction-LC–MS/MS) was applied for the determination of catecholamine and steroid hormone levels. Proteins were removed from plasma samples by precipitation. Subsequently polar and non-polar fractions were separated for both GC–MS and LC–MS/MS analysis by adding water and a mixture of ethanol and dichloromethane. For GC–MS analysis, the non-polar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The non-polar and polar fractions were further derivatized with O-methyl-hydroxylamine hydrochloride and pyridine to convert oxo-groups to O-methyl-oximes and subsequently with a silylating agent before analysis (Roessner et al., 2000). For LC–MS analysis, both fractions were reconstituted in appropriate solvent mixtures. HPLC was performed by gradient elution using methanol/water/formic acid on reversed phase separation columns. Mass spectrometric detection technology was applied which allows target and high sensitivity MRM (Multiple Reaction Monitoring) profiling in parallel to a full screen analysis (patent WO2003073464). For GC–MS and LC–MS/MS profiling, data were

Table 1
Rationale for the dose settings in the described 4 weeks Wistar rats study with administration of DBP and DEHP via the diet.

Dose level (ppm)	Expected toxicity	Purpose of investigation
Control	None	
3000 DEHP	Reduced body weight, liver toxicity, mild testicular toxicity	Metabolome at toxic dose level
150 DBP	No Observed Adverse Effect Level (NOAEL)	Metabolome at NOAEL, dose response
1000 DBP	Minimal toxic effects (liver)	Metabolome at Low Effect Level (LOEL), dose response
7000 DBP	Reduced body weight, liver toxicity, mild testicular toxicity	Metabolome at toxic dose level, dose response
150 DBP + 3000 DEHP	Only DEHP toxicity	Any effects of NOAEL DBP dose on DEHP metabolome profile?
1000 DBP + 3000 DEHP	DEHP + minimal DBP toxicity	Effects of LOEL DBP dose on DEHP profile
7000 DBP + 3000 DEHP	Combined toxic effects of DBP and DEHP assumed additivity	Additivity of effects of DEHP and DBP at toxic dose level on metabolome

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