



National Institute for Public Health  
and the Environment  
*Ministry of Health, Welfare and Sport*

## **Risk assessment of substances in combined exposures (mixtures)**

RIVM Letter report 2015-0189  
Y. Staal | L. van der Ven





National Institute for Public Health  
and the Environment  
*Ministry of Health, Welfare and Sport*

## **Risk assessment of substances in combined exposures (mixtures)**

RIVM Letter report 2015-0189  
Y. Staal | L. van der Ven

## Colofon

© RIVM 2015

Parts of this publication may be reproduced, provided acknowledgement is given to: National Institute for Public Health and the Environment, along with the title and year of publication.

Dr. Yvonne Staal, (author) RIVM  
Dr. Leo van der Ven, (author) RIVM

Contact:  
Dhr. drs. N.J.C. van Belle

This investigation has been performed by order and for the account of VWS, within the framework of project RBB

This is a publication of:  
**National Institute for Public Health  
and the Environment**  
P.O. Box 1 | 3720 BA Bilthoven  
The Netherlands  
[www.rivm.nl/en](http://www.rivm.nl/en)

## Publiekssamenvatting

### **Risicobeoordeling van mengsels van chemische stoffen**

Bij de risicobeoordeling en regulering van chemische stoffen wordt uitsluitend rekening gehouden met de schadelijke effecten van afzonderlijke stoffen. In de praktijk wordt de mens echter blootgesteld aan talloze stoffen tegelijk ('mengsels'). Dit gebeurt vooral via voedsel, maar kan ook op andere manieren zoals door inademing of via de huid. Geringe, nog onschadelijke effecten van afzonderlijke stoffen kunnen schadelijk worden als ze bij elkaar worden opgeteld. Verschillende nationale en internationale regelgevende instanties, waaronder de Europese Commissie en de Wereldgezondheidsorganisatie (WHO), hebben onderkend dat de risico's van deze gelijktijdige blootstelling aan verschillende stoffen moeten worden onderzocht.

Het RIVM heeft daarom in kaart gebracht welke initiatieven de afgelopen jaren zijn ondernomen om methodieken te ontwikkelen die de mogelijke gezondheidsrisico's van mengsels weergeven. Eén daarvan is afkomstig van de Europese Voedsel Autoriteit (EFSA) waaraan het RIVM bijdraagt met modelberekeningen. De EFSA-methode bevat echter nog veel onzekerheden en is specifiek ontworpen voor effecten van mengsels van bestrijdingsmiddelen die via voedsel worden opgenomen. Het is vooralsnog onduidelijk of de methode ook geschikt is voor andere stofgroepen zoals allergenen, en stoffen die niet via voedsel, maar door de lucht of via de huid in het lichaam terechtkomen.

Momenteel wordt onderzocht hoe deze EFSA-methode kan worden verfijnd door de bestaande gegevens over blootstelling aan en schadelijkheid van stoffen aan te vullen met nieuwe metingen die zijn gebaseerd op celkweekmodellen. RIVM voert dit onderzoek in samenwerking met internationale partners uit.

Kernwoorden: mengsels van stoffen, risicobeoordeling, veiligheid



## Synopsis

### **Risk assessment of substances in combined exposures (mixtures)**

Presently, risk assessment and regulation of chemical compounds is based on adverse health effects of single compounds only. However, in daily life, people are exposed to many compounds simultaneously ('mixtures'). Exposure is mainly via food, but other routes are also occur, e.g. by inhalation or via the skin. In this way, minimal, non-adverse effects can add to adversity. Several regulatory bodies, at the national and international level, including the EU Commission and the World Health Organization (WHO) have acknowledged that the risks of such combined exposures should be investigated.

RIVM has made an inventory of recent initiatives to develop methods and strategies for the assessment of health risks of mixtures. One such a strategy was developed by the European Food Safety Authority (EFSA), to which RIVM contributed with model calculations. The EFSA strategy, however, still has many uncertainties and was specifically developed in the context of exposure to pesticides via the food. It is unclear whether the method also can be applied for other groups of chemicals such as allergens, and for other exposure routes.

Presently, it is investigated how this EFSA method can be refined, through addition of further data on exposure and toxicity as derived from cell culture models. This research is conducted by a consortium of RIVM and international partners.

Keywords: mixture toxicity, risk assessment, compound safety





## Contents

**Summary – 9**

**Samenvatting – 10**

**1 Introduction – 11**

**2 Risk assessment of combined exposure to multiple chemicals (WHO/IPCS) – 13**

**3 EU Scientific Committees opinion on Cumulative risk assessment (CRA) – 15**

**4 Cumulative risk assessment as proposed by EFSA – 17**

**5 EuroMix – 19**

**6 Challenges – 21**

**7 Conclusions – 23**

**8 References – 25**



## Summary

Exposure to multiple substances in a mixture needs to be addressed in risk assessment because the potential adverse health effects in the mixture may be higher than of individual substances. WHO/ICPS designed a general framework for cumulative risk assessment, based on a tiered assessment of both exposure and hazard of substances in the mixture. This framework was generally adopted, including by several EU scientific committees. Following EU regulation 396/2005, EFSA was ordered to develop a method for cumulative risk assessment of pesticides. The efforts of EFSA produced a pragmatic method that can be applied to cumulative risk assessment of chemicals in general. The method of EFSA is based on a system in which substances are grouped in practical units, according to their target organs, the so-called Cumulative Assessment Groups (CAGs). It is however anticipated that the EFSA method is open for refinement. In the EFSA opinions on this subject it was highlighted that for many chemicals information is lacking, e.g. on mode of action, and refinements might be used in future risk assessment when such information becomes available.

This EFSA model in turn will be further developed and refined in the EU-Horizon2020 project Euromix, which started in May 2015. In this project, as well as in parallel initiatives, *in silico* and *in vitro* methods will be used to predict the cumulative effects of substances belonging to the same CAG for the *in vivo* situation. The EuroMix approach will make use of *in vitro* and *in vivo* testing, consider toxicokinetics, toxicodynamics, and dose-response relations of single substances in a mixture. These predictions will be made using a model that describes all sequential events between the initial interaction of a substance with the biological system and the apical toxicological phenotype (i.e. the Adverse Outcome Pathways model). Predictions will then be verified in an experimental *in vivo* animal model and in an epidemiological cohort.

This methodology will thus combine a mechanism-based quantitative methodology for hazard characterization incorporated with an advanced tool for exposure assessment. The effect of the methodology will be to increase the efficiency and effectiveness of safety evaluations of the impact of mixtures on human health with regard to the costs and number of laboratory animals used. Stakeholders such as WHO and EFSA will be involved in the progress of the project, to ensure future implementation of the developed tools.

Challenges remain regarding refinement of exposure and toxicity data of chemicals, and applicability to other groups of chemicals than pesticides, and combined routes of exposure.

## Samenvatting

Blootstelling aan mengsels van stoffen is een probleem in de risicobeoordeling omdat schadelijke effecten op de gezondheid in een mengsel groter kunnen zijn dan van stoffen afzonderlijk. WHO/ICPS heeft een algemene werkwijze ontworpen voor de risicobeoordeling van mengsels van stoffen. Deze werkwijze, die gebaseerd is op een stapsgewijze beoordeling van zowel blootstelling als schadelijkheid van stoffen in een mengsel, is breed geaccepteerd, o.a. door verschillende wetenschappelijke commissies van de EU. Volgens EU regulering 396/2005 werd EFSA verplicht om een praktische methode te ontwikkelen voor cumulatieve risk assessment van bestrijdingsmiddelen. Met inachtneming van de door de WHO opgestelde principes heeft dat een concrete, zelfstandige methodiek opgeleverd die ook voor andere mengsels dan pesticiden toepasbaar is. De EFSA methode behelst een systeem waarbij stoffen op basis van schadelijke effecten in eenzelfde orgaan gegroepeerd worden in overzichtelijk eenheden, de zogenaamde Cumulative Assessment Groups (CAGs). Deze door EFSA voorgestelde werkwijze wordt verder praktisch uitgewerkt en verfijnd in het EU-Horizon2020 project EuroMix, dat gestart is in mei 2015. In dit en in parallelle projecten worden *in silico* en *in vitro* methoden gebruikt om cumulatieve effecten van gegroepeerde stoffen voor de *in vivo* situatie te voorspellen, rekening houdend met toxicokinetiek, toxicodynamiek, werkingsmechanisme en dosis-effect relaties van afzonderlijke stoffen in een mengsel. Deze voorspellingen zullen zoveel mogelijk opgezet worden langs de lijnen van een model dat alle stappen beschrijft tussen de eerste interactie van een stof met biologisch systeem en het uiteindelijke toxicologische effect (het zogenaamde Adverse Outcome Pathways model). De toxicologische voorspellingen worden vervolgens geverifieerd in een *in vivo* model en in een epidemiologisch cohort. Het EuroMix project moet een instrumentarium opleveren waarmee de evaluatie van het gezondheidsrisico voor de mens van stoffen in een mengsel op een efficiënte en effectieve manier kan worden uitgevoerd. Belanghebbende organisaties zoals WHO en EFSA worden nauw bij de voortgang van het project betrokken om de toekomstige toepassing zoveel mogelijk te waarborgen. Er is aanvullend onderzoek nodig om noodzakelijke gegevens over chemische stoffen te verkrijgen en om de toepasbaarheid van de EFSA methode voor andere groepen van chemische stoffen dan bestrijdingsmiddelen en voor andere blootstellingsroutes te valideren.

## 1 Introduction

Every day, humans are exposed to multiple chemical substances via multiple routes of exposure: diet, inhalation and dermal contact. Such substances may exert toxic effects, which could accumulate in case exposure to the different substances occurs simultaneously. Accumulation of toxic effects is not addressed in current risk assessment, which only considers safe exposure levels of individual substances. It is generally acknowledged that combined exposures potentially can lead to cumulative effects, but no validated strategy for cumulative risk assessment (CRA) is available yet. The need to address combined exposures to mixtures of substances and their combined risk is identified as a problem by various international bodies involved in public health, like the International Programme on Chemical Safety of the World Health Organization (WHO/IPCS), EU-DG SANTE (Health and Food Safety), the European Food Safety Authority (EFSA), Codex Alimentarius, and the Environmental Protection Agency of the USA (US-EPA) (EFSA 2013b). It appears that opinions of these bodies in the issue overlap, and that the development of strategies to address combined exposures largely run in parallel. Here we summarize some major conclusions from these bodies, identified knowledge gaps, and preferred proposals for a strategy to address hazard and risk of combined exposures.



## 2 Risk assessment of combined exposure to multiple chemicals (WHO/IPCS)

The WHO/IPCS proposed a framework to aid risk assessors in identifying priorities for risk management for applications where co-exposure to multiple substances is expected (Meek et al, 2011). This framework is based on a hierarchical (phased) approach that involves integrated and iterative consideration of exposure and hazard at increasing level of detailed information (see Fig. 1). This tiered approach has the potential to considerably reduce the resources required to assess exposure to mixtures of substances. This framework led to a general approach for risk assessment of combined exposure to multiple substances that can be adapted to the need of specific users. The initial tier begins with simple but conservative assumptions for both hazard and exposure. These assumptions are refined and replaced with increasingly detailed data and models, but only if necessary. Therefore, if there is no cause for concern based on the assessment of a tier, no further resources are invested. However, if the initial assessment indicates an excessive risk, then the assessment is refined, incorporating more and more accurate models. At any tier, the outcome of margin of exposure analysis can be risk management, no further action, generation of additional data or further assessment. The evaluation of the adequacy of the margin of exposure is dependent on the actual purpose and/or (legal) framework for which the assessment is performed. This approach is a theoretical approach, which requires guidance on the practical implementation.

### Problem Formulation for Combined Exposure Assessment

- *What is the nature of exposure?*
- *Is exposure likely, taking into account the context?*
- *Is there a likelihood of co-exposure within a relevant timeframe?*
- *What is the rationale for considering compounds in an assessment group?*



### Example Tiered Exposure and Hazard Considerations: Mixture or Component Based

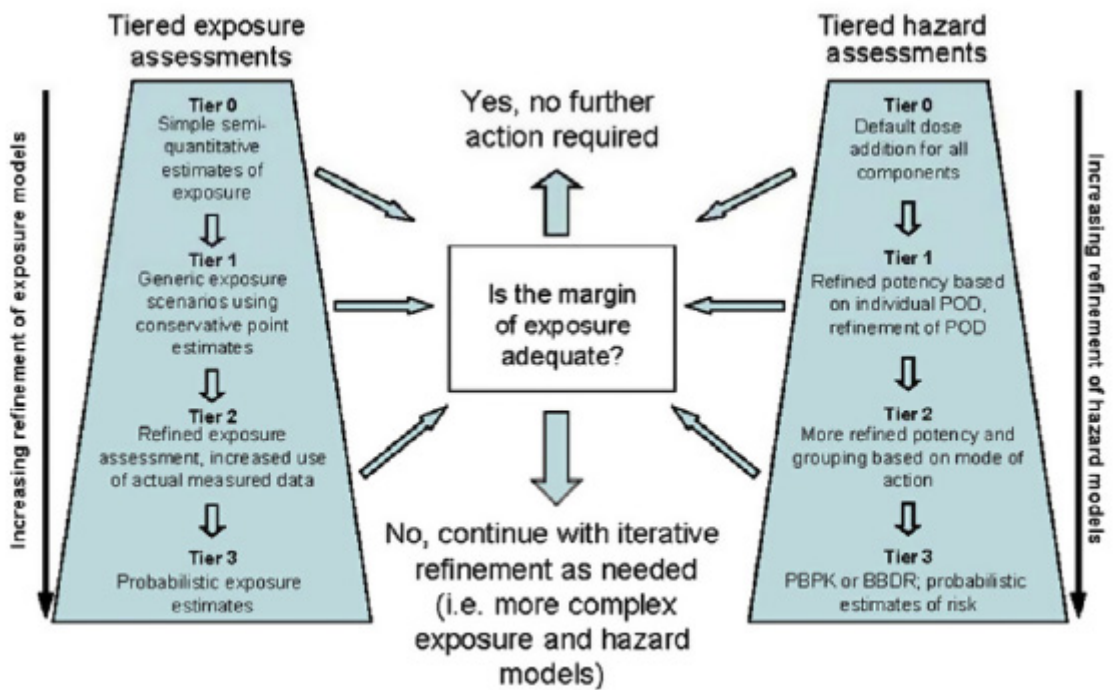


Figure 1: A conceptual representation of the framework (Meek et al, 2011)



### 3 EU Scientific Committees opinion on Cumulative risk assessment (CRA)

In 2011, the EU Scientific Committees on Health and Environmental Risks (SCHER), on Emerging and Newly Identified Health Risks (SCENIHR), and on Consumer Safety (SCCS), jointly approved an opinion, which concluded that chemicals could exert joint toxic actions, which may result in combination effects that are larger than the effects of each component applied singly (SCCS, SCHER, SCENIHR, 2012). These effects, which should be valid anyway for substances with *similar mode of action*, can be described by *dose/concentration addition*. For substances with *different modes of action (independently acting, IA)*, no robust evidence is available that exposure to a mixture of such substances is of health or environmental concern if the individual substances are present at or below their zero effect levels. *Interactions* (including *antagonism, potentiation, and synergy*) usually occur at medium or high dose levels (relative to the lowest effect levels). At low exposure levels, they are either unlikely to occur or are toxicologically insignificant. In view of the almost infinite number of possible combinations of substances to which humans and environmental species are exposed, some form of *initial filter* to allow a focus on mixtures of potential concern is necessary.

With regard to the assessment of chemical mixtures, a major knowledge gap is the lack of exposure information and the rather limited number of substances for which there is sufficient information on their mode of action. Currently, neither there is an agreed inventory of mode of actions, nor a defined set of criteria how to characterize or predict a mode of action for data-poor chemicals. If no mode of action information is available, the dose/concentration addition method should be preferred over the independent action approach. Prediction of possible interaction requires expert judgement and hence needs to be considered on a case-by-case basis. This opinion also adopted the decision framework proposed by WHO/IPCS (Meek 2011; SCCS, SCHER, SCENIHR, 2012).



## 4 Cumulative risk assessment as proposed by EFSA

Following EU regulation 396/2005, EFSA was ordered to develop a method for cumulative risk assessment of pesticides. EFSA thus proposed a more operational and practical strategy, which is applicable for cumulative effects of substances in general (EFSA 2013b).

### *General principles*

In the first place, EFSA adopted generally accepted principles related to cumulative risk assessment (e.g. Borgert *et al*, 2004), and indicated above. The most straightforward design is to assess cumulative risk on the consideration of exposures (relative concentrations/doses of substances in the mixture) or effects (relative toxic potency of substances in the mixture) for combinations of substances with similar mode of action. Alternatively, in a more complex design which also includes dissimilar modes of action, approaches are based on the assumption of dose additivity (where substances are considered to be toxicological similar), response additivity (where substances are considered to act independently), or interaction (where effects of combined exposure to components are expected to be greater than or less than those based on the assumption of dose additivity, i.e. respectively potentiation/synergism and antagonism). For dose additivity, approaches are normally based on summed indices of comparison of estimated exposure with hazard for components. Alternatively, they are based on summed estimated exposure to components adjusted by potency (the hazard index approach; for an example using anti-androgens, see Kortenkamp 2010).

### *Underlying considerations*

For interaction of pesticides, synergistic interactions between chemicals are rare and often occur only at high concentrations (Cedergreen 2014). This supports the validity of dose addition as a default assumption for mixtures. When considering mode of action, response addition may be the preferred approach for substances that have dissimilar modes of action. Use of the independent action model as an assessment concept for combination effects requires knowledge or demonstration that modes of action of individual substances in a mixture are strictly dissimilar; this puts demands on data quality of required input values that is rarely met in practice. Even in such cases for which independent action provided accurate predictions (particularly observed in the context of ecotoxicity), dose addition yielded the more conservative prediction. No example of the validity of independent action with mammals, mammalian cells or with multi-cellular organisms has been identified to date. Application of response addition method for predictions of toxicity requires detailed descriptions of effects, also in the low dose range, that are rarely available. Studies should be designed to establish quantitative relationships between dose (and time) dependency of responses, to improve mixture toxicity models. Additional information on pharmacodynamics, pharmacokinetics and dose response is required for adequate application of models for cumulative risk assessment. Similarly, it is important that information on the mechanism or mode of action is available to assess the effects of a mixture of substances. This

information could be refined using 'omics' techniques, even at low doses (Altenburger, 2012).

The differences between predicted mixture effects derived from dose addition and those derived from independent action are likely to be small. Furthermore, no case could be identified in which independent action provided a more conservative mixture effect prediction than dose addition. It was therefore concluded that dose addition is a sufficiently conservative approach to protect consumers' health.

#### *Pragmatic operational approach*

Altogether, EFSA recommended using cumulative risk assessment methods derived from dose addition, provided they produce a common adverse outcome. Secondly, to reduce the chemical universe to workable units, EFSA proposed a system of grouping substances that produce common adverse outcomes in the same target organ/system into so-called *cumulative assessment groups (CAGs)* (EFSA 2013a). Substances exhibiting dissimilar mode of action can potentially produce a variety of different, non-overlapping, toxic effects in different organs/systems. In such cases, it is difficult to identify a combination effect. Therefore, the considerations were restricted to combinations with dissimilar modes of action to substances that produce a common adverse effect on the same organ/tissue. Their combined effects should be assessed by using the concept of dose addition as a pragmatic and conservative default approach for the purpose of assessing cumulative risk in relation to maximum residue limits (MRL) setting or risk assessment of chemical mixtures in practice.

Some of the cumulative assessment groups may still contain a large number of pesticides. Further reduction of group sizes can be obtained by refinement of phenotypes within a CAG (level 1), producing "level 2" grouping, e.g. cholestasis, steatosis, etc, within the CAG "liver". EFSA further considered that even with a mixture of many pesticides affecting the same target organ/system, the majority of them might not contribute significantly to a given combined effect because exposure is very low and/or potency in relation to the considered effect is weak. Cumulative risks from actual exposure are likely to be driven mainly by a few pesticides in a mixture. Further grouping of chemicals according to their modes of action (level 3) and mechanism of action (level 4) may lead to further refinement within each CAG. Until now, EFSA adopted two CAGs, i.e. based on organ toxicity for the thyroid and for the nervous system. Some other CAGs, including liver and developmental toxicity, are under consideration for adoption in the coming year, and many more are to follow. RIVM contributed to the drafting of CAGs (Vv.Aa, 2013).

Some specific exposure and hazard related considerations feeding the EFSA model are difficult to verify, such as the pragmatic assumption that mixture effects will not arise if all components are below their individual effect levels, in this case due to difficulties in defining doses/concentrations without effect.

## 5 EuroMix

The most practicable methodology to address mixture toxicity is proposed by EFSA, and therefore this is being implemented in the Horizon2020 project EuroMix. This project started in May 2015 and is coordinated by RIVM. Two CAGs as drafted by EFSA were selected as cases to provide proof-of-principle for the concepts as put forward by EFSA, i.e. liver and developmental toxicity. Endocrine disruption, although not anticipated as an EFSA CAG, will be assessed as well, and the same is true for immunotoxicity, through a limited set of parameters. For this purpose, the EuroMix project approach is based on a high level of integration of available *in silico* and *in vitro* methods, which together should appropriately predict combination effects *in vivo*. This will be validated in dedicated *in vivo* studies, and a parallel verification study will be done in a human cohort. Selection of relevant substances is driven by exposure data and information on modes of action. An important asset is that the *in vitro* toolbox should be designed to provide input for existing adverse outcome pathways (AOPs), or to contribute to new AOPs, and that AOPs will be used to structure the predictions for the *in vivo* situation. The anticipated outcome of the EuroMix project is a validation of the EFSA concept for CRA, including supporting *in silico* and *in vitro* toolboxes for the studied CAGs. The effect of the methodology will be to increase the efficiency and effectiveness of safety evaluations of the impact of mixtures on human health with regard to the costs and number of laboratory animals used. Stakeholders such as WHO and EFSA will be involved in the progress of the project, to ensure future harmonized implementation of the developed tools.



## 6 Challenges

To further evaluate and validate this approach, EFSA identified essential knowledge gaps, resulting in the following recommendations:

- more research is required in mammalian systems to investigate the applicability of independent action;
- more knowledge is required on distribution of slopes of dose response curves in mammalian systems to distinguish cases for which independent action might provide the more conservative prediction;
- key knowledge on mode/mechanism of action with relevance to CRA should be enriched;
- more research is required on the possibility of toxicokinetic and toxicodynamic interactions, in order to better define determinants of interactions, e. g. of synergisms.

The EuroMix project, together with other initiatives, may be able to provide a proof-of-principle for practical application of the EFSA strategy for cumulative risk assessment. However, EFSA specifically addressed pesticides in food, and broadening to other groups of chemicals, such as allergens, and to other routes of exposure in humans (dermal, inhalatory) needs to be established.





## 7 Conclusions

Until now, there is no operational strategy to consider cumulative effects of combined exposures. Initiatives of several international bodies to address cumulative risk assessment produced conceptual, overlapping frameworks. Following these initiatives, EFSA proposed a more pragmatic, operational approach, based on so-called cumulative assessment groups. This EFSA approach holds the best promise for implementation in risk assessment, and is therefore supported by RIVM. Further refinement and validation of the EFSA strategy is however required, and this is the goal of the Horizon2020 project EuroMix, using a limited number of cumulative assessment groups. Altogether, EuroMix, as well as other initiatives, will combine a mechanism-based quantitative methodology for hazard characterization incorporated with an advanced tool for exposure assessment. The effect of the applied methodology will be to increase the efficiency and effectiveness of safety evaluations of the impact of mixtures on human health with regard to the costs and number of laboratory animals used. Several challenges remain to refine the proposed approach and for broadening to other groups of chemicals than pesticides, and other routes of exposure than through food.



## 8 References

- Altenburger R., Scholz S., Schmitt-Jansen M., Busch W., Escher B.I., *Mixture Toxicity Revisited from a Toxicogenomic Perspective*, Environ Sci and Technol, 2012, vol 46 p2508-2522
- Borgert C.J., Quill T.F., McCarty L.S., Mason A.M, *Can Mode of Action Predict Mixture Toxicity for Risk Assessment?*, Toxicol Appl Pharmacol, 2004, vol 201, p85-96
- Cedergreen N., *Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology*, PLoS One, 2014, vol 9, issue 5
- EFSA Panel on Plant Protection Products and their Residues (PPR), 2013a. *Scientific Opinion on the identification of pesticides to be included in cumulative assessment groups on the basis of their toxicological profile*. EFSA Journal 2013;11(7):3293.
- EFSA 2013b. *International Framework Dealing with Human Risk Assessment of Combined Exposure to Multiple Chemicals*. EFSA Journal 2013;11(7):3313.
- Kienhuis AS, Slob W, Gremmer ER, Vermeulen JP, Ezendam J. A dose-response modeling approach shows that effects from mixture exposure to the skin sensitizers isoeugenol and cinnamal are in line with dose addition and not with synergism. Toxicol Sci. 2015 Sep;147(1):68-74.
- Kortenkamp A, Faust M. Combined exposures to anti-androgenic chemicals: steps towards cumulative risk assessment. Int J Androl. 2010 Apr;33(2):463-74.
- Meek M.E., Boobis A.R., Crofton K.M., Heinemeyer G., Van Raaij M., Vickers C., *Risk Assessment of Combined Exposure to Multiple Chemicals: A WHO/IPCS framework*, Regul Toxicol and Pharmacol, 2011, vol 60, PS1-S14
- Scientific Committee on Consumer Safety (SCCS), Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), Scientific Committee on Health and Environmental Risks (SCHER). *Toxicity and Assessment of Chemical Mixtures*, European Union, 2012. 50 pp. Available online: [http://ec.europa.eu/health/scientific\\_committees/environmental\\_risks/docs/scher\\_o\\_155.pdf](http://ec.europa.eu/health/scientific_committees/environmental_risks/docs/scher_o_155.pdf).
- Vv.Aa. Toxicological data analysis to support grouping of pesticide active substances for cumulative risk assessment of effects on liver, on the nervous system and on reproduction and development. Supporting Publications 2013:EN-392. [www.efsa.europa.eu/publications](http://www.efsa.europa.eu/publications).

