

# 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

Anne S. Kienhuis\*, Wout Slob<sup>†</sup>, Eric R. Gremmer\*, Jolanda P. Vermeulen\*, Janine Ezendam<sup>\*,1</sup>

\*Centre for Health Protection and <sup>†</sup>Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment (RIVM), 3721 MA Bilthoven, The Netherlands

<sup>1</sup>To whom correspondence should be addressed at Centre for Health Protection, National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands. Tel: +31 30 274 3447. E-mail: janine.ezendam@rivm.nl.

## ABSTRACT

Currently, hazard characterization of skin sensitizers is based on data obtained from studies examining single chemicals. Many consumer products, however, contain mixtures of sensitizers that might interact in such a way that the response induced by a substance is higher than predicted in the hazard assessment. To assess interaction of skin sensitizers in a mixture, a dose-response modeling approach is applied. With this approach, it is possible to assess whether or not responses from mixtures of sensitizers can be predicted from the dose-response information obtained from individual chemicals using dose addition. We selected the skin sensitizers isoeugenol and cinnamal, frequently occurring together in consumer products, to be examined in an adjusted local lymph node assay (LLNA). Cell number and cytokine production (IL-10 and IFN- $\gamma$ ) of the auricular lymph nodes were measured as hallmarks of the skin sensitization response. We found that dose addition for these 2 skin sensitizers closely predicted the effects from mixtures of both chemicals across the broad dose range tested. Hence, isoeugenol and cinnamal show no synergistic effects in the LLNA. Therefore, hazard assessment and risk assessment of these substances can be performed without taking into account mixture exposure.

**Key words:** skin sensitization; mixture; dose response; synergism; dose addition; LLNA.

Allergic contact dermatitis is an adverse human health effect caused by dermal exposure to chemicals with skin sensitizing properties (Kimber *et al.*, 2002). The risk of sensitization is dependent on the exposure concentration and the potency of a skin sensitizer (Gerberick *et al.*, 2001). Hazard characterization of skin sensitizing chemicals is important in different European regulations for safety assessment of chemicals, such as Registration, Evaluation and Authorization of Chemicals (Regulation [EC] No 1907/2006) and the Cosmetics Directive (Regulation [EC] No 1223/2009). Hazard characterization is

predominantly based on animal studies testing a single substance. Besides hazard characterization, a quantitative approach has been developed that aims at estimating safe exposure levels for consumers. This quantitative risk assessment (QRA) can be used to determine maximum concentration levels that should not be exceeded in consumer products. Acceptable exposure levels are sensitization thresholds derived from a weight of evidence evaluation of all available data, including human. To account for intraindividual differences and uncertainties between experimental and real-life exposure,

sensitization assessment factors are applied (Api et al., 2008). The QRA is based on sensitization thresholds for an individual fragrance and it has not been developed for safety assessment of mixtures.

Safety assessments for skin sensitizers are thus based on data from individual sensitizers, but consumers will often be exposed to mixtures of sensitizers. Consumer products are known to contain mixtures of different sensitizers, eg, deodorants can contain a cocktail of up to 17 known allergenic fragrances (Rastogi and Johansen, 2007). Recent German surveys (Rastogi and Johansen, 2007; Uter et al., 2013, 2014a,b) found mixtures of established skin sensitizers, including fragrances, preservatives and ultraviolet-filters, being present in cosmetic products. Additionally, consumers may use several products at the same time on the same skin area, leading to aggregate exposure (Hall et al., 2011). To assess human safety accurately, it is important to know whether exposure to a mixture of sensitizers leads to interactions that influence the response to a single substance.

The usual way to predict responses from mixtures is by dose addition (Berenbaum, 1985). In case chemicals show interaction, the response from mixtures may be larger or smaller than that predicted by dose addition. In case of skin sensitization, the immune response can only occur when an immunogenic hapten is formed together with the generation of a sufficient degree of epidermal danger signals (Martin et al., 2011; Natsch, 2010). The formation of haptens is unique for each chemical and due to this specificity; interaction unlikely to occur at this level. In contrast, the generation of epidermal danger signals, eg, oxidative stress and pro-inflammatory mediators, is nonspecific and can result from co-exposure by other chemicals, including irritants (Smith et al., 2002) and skin sensitizers (Esser et al., 2012). Therefore, exposure to a mixture of skin sensitizers might lead to interaction due to a higher level of danger signals being generated by the mixture.

In the past, animal studies demonstrated that combined exposure to a skin sensitizer and an irritant, specifically enhanced the adaptive immune responses induced by weak skin sensitizers or by strong skin sensitizers dosed at low, subirritant concentrations (Cumberbatch et al., 1993; De Jong et al., 2002). Apparently, strong skin sensitizers are able to generate sufficient danger signals if the exposure dose is sufficiently high. Experimental evidence for this phenomenon in case of mixture exposure to skin sensitizers has been found as well (Bonefeld et al., 2011; Jowsey et al., 2008). The effects of exposure to a mixture of 2 strong sensitizers was in the local lymph node assay (LLNA) by comparing empirically assessed mixture effects to the theoretical summation of responses of the individual sensitizers. Effect addition, however, is not an appropriate way of examining interaction of chemicals. Bonefeld et al. (2011) investigated a cocktail of 3 skin sensitizers, also in the LLNA. Results showed a more vigorous ear swelling and cell proliferation response upon exposure to mixtures of fragrance allergens compared with exposure to each fragrance individually. It was not established whether these effects were larger than predicted by dose addition.

Current studies do not provide a clear answer to the question whether responses from mixtures of sensitizers can be predicted from the dose-response information obtained from individual chemicals. The aim of this study is to assess whether the response of a given set of skin sensitizers can be predicted by dose addition. We performed a statistical dose-response modeling approach, thereby using results from the adjusted LLNA upon exposure to the skin sensitizers isoeugenol (IE) and cinnamal (CA), 2 fragrances frequently occurring together in consumer products.

## MATERIALS AND METHODS

In this study, a dose-range finding experiment and a mixture experiment are performed. For clarity, we chose to this study design, statistical analysis and results of both experiments in chronological order.

**Animals.** Female BALB/cOlaHsd mice, aged 8–12 weeks, were obtained from Harlan, The Netherlands. Mice were acclimatized for 7 days before the start of the study. Animals were bred under specified pathogen-free conditions. During the experiments, animals were housed in macrolon III cages, under conventional conditions in light-, humidity-, and temperature-controlled rooms. Food and tap water were available *ad libitum* during the whole experiment. Experiments were conducted under ethical review in accordance with Dutch law.

**Chemicals.** IE (2-Methoxy-4-propenylphenol, purity 99%) and CA (3-phenylprop-2-enal, purity  $\geq 95\%$ ) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Chemicals were dissolved in 4:1 acetone/olive oil (AOO). Control groups were treated with vehicle, AOO, alone. The same batch of test material was used throughout the study (experiment 1, experiment 2).

**Adjusted LLNA.** Groups of mice ( $n=4$ ) were treated with 25  $\mu$ l test chemical or vehicle (AOO) on the dorsum of both ears. Animals were exposed daily, for 3 consecutive days. At day 5, animals were sacrificed and the auricular lymph nodes were excised and pooled. Cell suspensions were prepared by disaggregation through cell strainers and collected in RPMI medium containing 10% fetal calf serum and 100 units/ml penicillin/streptomycin. For each mouse, the number of lymphocytes was determined. Lymphocytes were plated in a 96-well plate at  $4 \times 10^5$  cells/well and exposed for 24 h to 5  $\mu$ g/ml concanavalin A (ConA) to stimulate cytokine proliferation. The levels of IFN- $\gamma$ , IL-2, and IL-10 were determined using BioPlex (Biorad, Veenendaal, The Netherlands) (van der Veen et al., 2013).

**Experimental design dose-range finding experiment.** To make an efficient selection of dose combinations in the mixture experiment, an estimate of the relative potency factor (RPF) is needed (see previous section). Both chemicals had already been tested earlier in 2 separate LLNA studies, which seemed to indicate that IE might be slightly more potent than CA (Kimber et al., 1991; Wright et al., 2001). However, a difference in estimated potency might be biased by study differences. Therefore, we decided to first examine both chemicals in a single study, so as to obtain a more reliable RPF estimate. Based on the earlier separate studies (Kimber et al., 1991; Wright et al., 2001), doses for IE and CA were chosen as indicated in Table 1. All dose groups consisted of 4 animals, while the 2 control groups (1 for each chemical) each consisted of 8 animals.

TABLE 1. Doses and Group Sizes for Each Dose Group

| IE dose (% in AOO)       | 0 | 1   | 2   | 4   | 8  | 16 |
|--------------------------|---|-----|-----|-----|----|----|
| Cinnamal dose (% in AOO) | 0 | 1.9 | 3.8 | 7.5 | 15 | 30 |
| Group size <sup>a</sup>  | 8 | 4   | 4   | 4   | 4  | 4  |

<sup>a</sup>Related to each of the above 2 cells in the table.

Statistical analysis.. Using the PROAST software ([www.proast.nl](http://www.proast.nl)), the data were statistically analyzed by fitting the following 4-parameter exponential dose-response model to the data:

$$y = a\{c - (c - 1)\exp(-bx^d)\} \quad (1)$$

where  $y$  denotes the response, and  $x$  the dose. The constants  $a$ ,  $b$ ,  $c$ , and  $d$  in expression (1) are parameters to be estimated by fitting the model. Parameter  $a$  reflects the response in the controls, parameter  $b$  relates to the potency of the test chemical, parameter  $c$  reflects maximum response, and parameter  $d$  reflects the steepness of the curve. This model adequately describes a large variety of toxicological dose-response data, including LLNA dose-response data (Slob and Setzer, 2014). The dose-response model was fitted to the data of both chemicals combined, by including the factor “chemical” as a covariate. In this way, the statistical analysis can detect which of the parameters  $a$ ,  $b$ ,  $c$ , and  $d$  differ significantly between the chemicals (Slob, 2002). Parameters that are not significantly different may be kept the same for both chemicals in the final analysis.

Instead of the model given by expression (1), we applied an equivalent model where the potency parameter  $b$  is replaced by a parameter that represents an equipotent dose; for instance, the dose where the response is 3-fold higher than the background response. This dose could also be called a benchmark dose (BMD) with a benchmark range (BMR) of 200%. By fitting this model, a confidence interval for that BMD can be obtained using maximum likelihood theory (Moerbeek et al., 2004). To obtain a confidence interval for the relative potency factor (RPF), the model was fitted allowing the BMD to be different between the 2 chemicals. Furthermore, the BMD for one of the chemicals was expressed as RPF times the BMD of the other chemical:  $BMD_{ie} = RPF \times BMD_{ca}$ . In this way, the RPF is a parameter in the model, for which a confidence interval can be calculated using the maximum likelihood method just mentioned.

**Dose addition and statistical approach.** The statistical dose-response modeling approach used in this study aims to answer the question of whether effects from mixtures of 2 chemicals can be predicted by dose addition. To predict the response induced by a mixture consisting of dose  $dA$  of chemical A and dose  $dB$  of chemical B, the following steps were applied

1. Determine the dose-response of chemical A and that of chemical B. Establish if both dose-responses are approximately parallel on log-dose scale.
2. If it can be assumed that the dose-responses of A and B are parallel on log-scale, calculate the relative potency factor (RPF); note that  $\log(RPF)$  is the horizontal distance between both dose-response curves on log-dose scale.
3. Calculate the dose of chemical A that is equipotent to  $dB$ , by multiplying  $dB$  by the RPF.
4. Add the original  $dA$  to the dose calculate in step 3.
5. Read of the response at the added dose using the dose-response of chemical A.

The crucial question therefore is whether the response observed after applying a given dose mixture is close to the one predicted in step 5, using dose-response data for the individual chemicals. To establish if dose addition applies, the RPF needs to be estimated with sufficient precision, ie, the study design needs to result in good dose-response information of A and B separately. To establish if dose addition applies globally (over the whole dose range), various dose mixtures, yielding various levels of response, are needed in the study design.

In practice, the 5 steps can be combined in a single experiment, by using a study design that includes doses related to the individual chemicals (marginal doses) as well as various mixtures of doses (mixture doses). Then, a dose-response model, which includes the RPF as a model parameter, is fitted to all these dose-response data combined. In this way, the RPF is efficiently estimated using both the responses from the individual doses and from the dose mixtures. If this fitted model (assuming dose addition) closely describes the responses of the mixtures, it is confirmed that the mixture responses can be closely predicted. The statistical error in the data is taken into account by calculating confidence intervals for the responses in each (mixture) dose group.

It may be noted here that we purposely do not follow the more usual statistical approach of testing a null hypothesis by a significance test. The question is if dose addition can predict responses from mixtures *approximately or close enough*, and not if dose addition holds *perfectly*. Furthermore, in a real-life experiment, dose addition cannot perfectly hold, as real data always contain nonrandom errors, while random errors make it impossible to establish perfect dose addition even if nonrandom errors did not exist. Therefore, the results from the analysis will be expressed visually, while the statistical errors in the data are accounted for by confidence intervals. Such visual output provides valuable information, such as an indication of the precision by which mixture responses were predicted in the particular dataset assuming dose addition applies, or revealing a particular pattern in deviations from dose addition if it does not apply.

## RESULTS

### Dose-Range Finding Study of the Individual Chemicals

The results of the dose-range finding experiment with various separate doses for both chemicals are plotted in Figure 1. The parameters  $IFN\gamma$  and cell number show steadily increasing dose responses for both chemicals. The data were statistically analyzed by fitting the 4-parameter exponential model with chemical as a covariate. None of the 4 model parameters were found to differ significantly between the 2 chemicals, for both  $IFN\gamma$  and cell number. This implies that both chemicals could be described with exactly the same dose-response curve. Nonetheless, we also fitted the model by expressing the potency of CA as an RPF times the potency of IE (where potency was expressed as the chemical-specific BMD, ie, the where the response increased by a factor of 3 compared with background, ie,  $BMD_{ca} = BMD_{ie} / RPF$ ). This model, fitted to both response data, is shown in Figure 1. The 90%-confidence intervals for the RPF were (0.51, 1.15) and (0.32, 1.19) for  $IFN\gamma$  and cell number, respectively. These RPF confidence intervals indicate that IE might be somewhat more potent than CA, but the difference is not statistically significant, because the confidence intervals include the value of 1.

### Effects of Mixture Exposure to Cinnamal and IE in the LLNA

Using the information on the relative potency of the 2 chemicals as provided by the dose-range finding study, an efficient choice of dose combinations can be made to be used in a mixture experiment aiming to establish whether dose addition applies or not. We applied the approach described previously (Bosgra et al., 2009), which can be visualized by an isobole plot, as illustrated in Figure 2. In this plot, the isoboles are straight lines reflecting all dose combinations that will result in the same

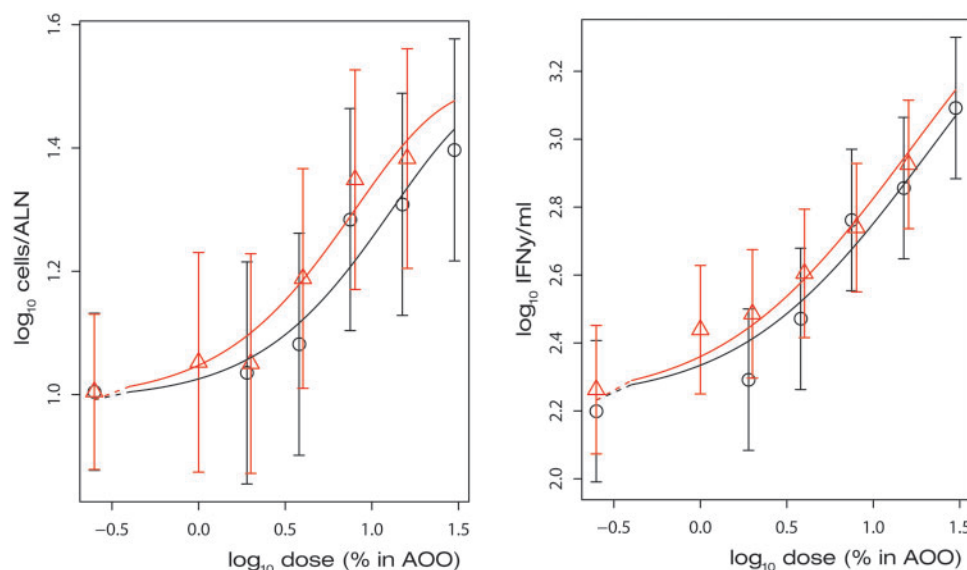


FIG. 1. Geometric group means of IFN $\gamma$  (right panel) and number of cells per auricular lymph node (left panel) as a function of dose, related to CA (circles, lower curve), and to IE (triangles). The vertical whiskers are 95% confidence intervals for the responses. The curves represent the fitted 4-parameter exponential model, see expression (1). Parameters  $a$ ,  $c$ , and  $d$ , as well as the within group variance ( $var$ ) were assumed the same in both chemicals.

response, if dose addition applies. In particular, the response will be the same as that from one of the marginal doses related to one of the individual chemicals (indicated by the filled marks). The marginal doses on the same isobole are equivalent doses by taking the RPF into account.

By choosing dose combinations from various isoboles, various response levels will be covered in the mixture experiment. This is important as the assertion that dose addition applies is practically useful only if it holds globally (for the whole dose range, or equivalently, for the whole response range). To achieve various response levels associated with the applied dose mixtures, the dose-response relationships of the individual chemicals need to be approximately known, as well as the RPF. To ensure that the isoboles for these 2 chemicals are approximately known, and dose mixtures can be derived that would most likely result in the various response levels specified, a range finding study may need to be performed first (as we did in this study). In this way, the available information to be realized from the mixture experiment will be maximized. The open circles in Figure 2 illustrate possible dose combinations that might be selected for an intended mixture experiment, which in this illustrative figure represent dose mixtures where both equal (1 circle in the middle of the line) and unequal dose combinations (2 circles on the same line) are covered.

As the dose-range finding experiment with IE and CA resulted in an estimated RPF that was close to unity, we created an isobole plot as in Figure 2 assuming that both chemicals were equally potent ( $RPF = 1$ ). Furthermore, because the dose-range finding experiment resulted in observed responses that were gradually increasing from small to relatively large, the applied doses in the first study could well serve as marginal doses in the mixture experiment. However, the mixture experiment should also include mixtures of IE and CA, and to keep the experiment within practical limits, we decided to omit various marginal doses. As Table 2 shows, we applied increasing marginal doses that alternated between both chemicals. In this way, reasonable information for the marginal dose responses for IE and CA will be obtained with a limited number of dose groups.

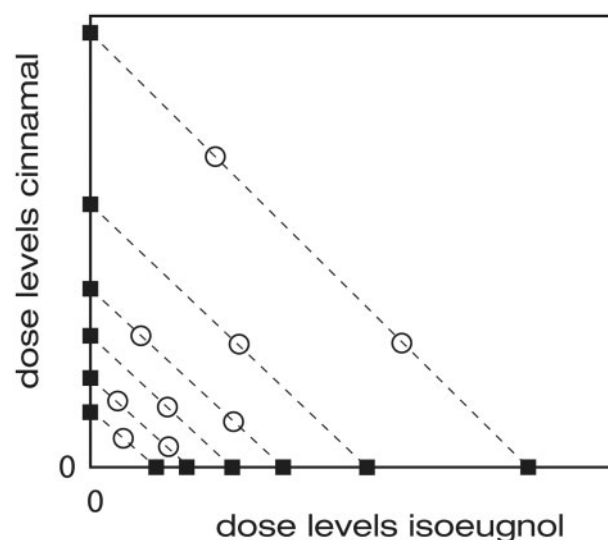


FIG. 2. Hypothetical representation of isoboles when dose addition applies. In this representation, the response of any dose-combination can be predicted based on the response upon treatment with an individual dose (black dot on the x- or y-axes).

Regarding the mixture doses, we started with the combination of 2% + 2% dose units as the lowest dose mixture. The first experiment showed that doses lower than 4% (single chemical) resulted in responses of the same order of magnitude as the statistical noise in the data, which would make them hardly informative regarding dose addition (unless violated by strong synergism). The next mixture doses were chosen such that their sums were similar to the increasing marginal doses (aiming at similar response levels), with some mixtures consisting of 2 equal doses, others consisting of 1 larger and 1 smaller dose (see Table 2).

#### Dose-Response Modeling of the Mixture Exposure

The observed responses related to the marginal doses and the dose mixtures were statistically analyzed by fitting the same

**TABLE 2.** Marginal Doses and Mixture Doses as Used in the Mixture Experiment

| IE             | Cinnamal | Group Size <sup>a</sup> |
|----------------|----------|-------------------------|
| Marginal doses |          |                         |
| 0              | 0        | 8                       |
| 1              | 0        | 4                       |
| 0              | 2        | 4                       |
| 4              | 0        | 4                       |
| 0              | 8        | 4                       |
| 16             | 0        | 4                       |
| 0              | 32       | 4                       |
| Dose mixtures  |          |                         |
| 2              | 2        | 4                       |
| 4              | 4        | 4                       |
| 4              | 12       | 4                       |
| 12             | 4        | 4                       |
| 16             | 16       | 4                       |
| 24             | 8        | 4                       |
| 8              | 24       | 4                       |

<sup>a</sup>Related to each row in the table; all doses are expressed as % in AOO.

4-parameter exponential model as in the dose-range finding experiment that applied only individual doses of both chemicals (marginal doses). Various approaches are possible to examine dose addition. One approach is to first estimate the RPF from the marginal doses (as in Figure 1), and then predict the response for each dose mixture assuming dose addition. The predictions may then be plotted against the observed responses, and if dose addition applies, this should result in a linear scatter plot (Bosgra et al., 2009). We used a different approach that more efficiently uses the information in the data, and is easier to apply, with suitable software, such as PROAST. In this approach, the dose  $x$  in expression (1) is simply replaced by  $\text{dose}_{\text{CA}} + \text{RPF dose}_{\text{IE}}$ , so that both the marginal and mixture doses can be handled in the same fit (note that for marginal doses the other dose is set at zero). Further, RPF is considered as an additional model parameter to be estimated in fitting the model. After having fit the model, the observed responses are plotted against  $\text{dose}_{\text{CA}} + \text{RPF dose}_{\text{IE}}$ , while making visible which responses related to either single chemical or to the mixtures. If dose addition applies, the responses related to the dose mixtures should, in general, be as close to the fitted dose-response curve as the responses related to the single dose. In evaluating this visual plot, the confidence intervals around the responses may be helpful: when dose addition applies, the fitted curve should intersect most of them.

Instructions to perform this analysis in the PROAST software are provided as supplementary data. The observed responses related to the marginal doses and to the dose mixtures are shown in Figure 3 for 3 immunological parameters. In all cases, the confidence intervals for the responses at the mixtures intersect the fitted curve, which represents the expected response if dose addition applies. This implies that the observed responses from the mixtures are in line with the predicted responses based on dose addition.

The RPF confidence intervals for the various immunological parameters, estimated in either of the 2 experiments, are summarized in Table 3. No differences in estimates are observed between both studies, but it seems that the parameters differ somewhat in RPF.

## DISCUSSION

In this study, we used a dose-response modeling approach to establish whether responses from a mixture of 2 skin sensitizers can be predicted from the dose-response information from the individual chemicals. The fragrances IE and CA were selected for this study, because these 2 fragrances are sensitizers of moderate potency and frequently occur together in consumer products. It has been hypothesized that synergism is most likely to occur with weak to moderate skin sensitizers present in the mixture (Jowsey et al., 2008; Bonefeld et al., 2011). Our study did not support this hypothesis, because the dose response induced by the mixture of moderate skin sensitizers follows from the independent responses induced by the individual sensitizers. If the same holds true for other combinations of skin sensitizers including strong sensitizers is unknown. Most probably, strong skin sensitizers will generate sufficient danger signals in the skin and as such, inflammation induced by other skin sensitizers present in the mixture will not add up to this.

Synergism of skin sensitizers has been previously suggested based on other LLNA studies (Jowsey et al., 2008; Bonefeld et al., 2011). However, in both studies, conclusions were based on comparison of empirical data with a theoretical summation of responses of single substance and were not assessed using dose-response modeling. Summation of effects is not an appropriate way of examining interaction between chemicals. Specifically, Jowsey et al. (2008) showed that a combination of the strong skin sensitizers methyldibromo glutaronitrile and para-phenylene diamine induced a slightly higher response in the LLNA than was predicted from summing the effects induced by the individual sensitizers, especially at subirritant concentrations. This effect was, however, not reproducible in a second experiment. Furthermore, a thorough statistical analysis was not possible, because the lymph nodes were pooled for each experimental group, rather than using the individual results (Jowsey et al., 2008). Bonefeld et al. (2011) found evidence for an increased response in both the sensitization and elicitation phase for a mixture of 3 fragrances compared with responses induced by the individual responses. This study did not allow for the assessment of dose addition or interaction (Bonefeld et al., 2011).

Our primary interest in this study was to elucidate if dose addition applies in the induction phase, because this is relevant for primary prevention of sensitization in the general population. The QRA as developed by fragrance industry (Api et al., 2008) is an instrument used to derive safe levels for a single ingredient in a consumer product. Because our study shows no synergism for these sensitizers the QRA can be applied to the individual fragrances for consumer products that contain the mixture. It should be emphasized that in the LLNA the individual immune responses induced by IE and CA cannot be distinguished at the level of lymphocyte proliferation, because the LLNA only measures the induction phase.

In the LLNA, we demonstrate that dose addition occurs, but it is important to note that this does not imply that the specific immune responses induced by these skin sensitizers will also behave according to dose addition. The reason is that priming of the immune system is highly specific and unique for the individual skin sensitizers. The lack of interaction between IE and CA, however, shows that there is no interference at the level of epidermal danger signals that could enhance the hapten-specific immune response. For this reason, data of a single substance can be used for hazard characterization and risk assessment without taking into account mixture exposure.

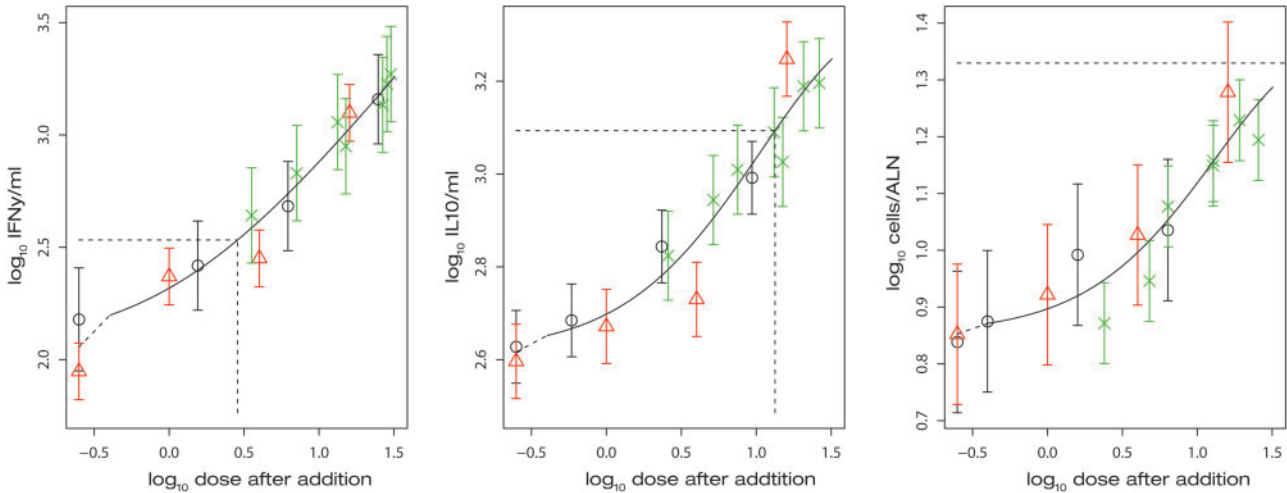


FIG. 3. Observed responses (log-scale) from marginal doses (triangles: IE, circles: CA) and from dose mixtures (crosses) with 95% confidence intervals. The (log-)dose on the x-axis is given by  $d_{IE} + RPF \cdot d_{CA}$ , with the value of RPF of 0.78 in left panel, 0.29 in center panel, and 0.20 in right panel. Note that the lowest dose represents the controls, which in reality are located at minus infinity on log-scale (hence the dashed curve at the lower end). The horizontal dashed line indicates a 3-fold increase relative to background, the vertical dashed line the associated dose (Critical Effect Dose = BMD) of 2.86 in left panel, 13.4 in center panel, and 50.7 in right panel (which is just outside the plot).

TABLE 3. RPF Confidence Intervals for Various Immunological Parameters, Estimated in the Dose-Range Finding and in the Mixture Experiment

|                  | Dose-Range Finding Experiment | Mixture Experiment |
|------------------|-------------------------------|--------------------|
| IFN $\gamma$ /ml | (0.51, 1.16)                  | (0.54, 1.14)       |
| IL10/ml          | (0.16, 1.36)                  | (0.20, 0.42)       |
| Cell number      |                               | (0.10, 0.35)       |

In conclusion, our study showed that no synergistic effects occur between IE and CA across a broad dose range in the LLNA. Our conclusion is restricted to IE and CA, but the dose-response modeling approach used in this study allows for testing of other sensitizers or more complex mixtures.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

FUNDING

This research was funded by the Dutch Ministry of Health, Welfare and Sports (VWS).

ACKNOWLEDGMENTS

The authors thank Prof. Dr Henk van Loveren for his valuable input in designing the experiments and critically reading this manuscript.

REFERENCES

Api, A. M., Basketter, D. A., Cadby, P. A., Cano, M.-F., Ellis, G., Gerberick, G. F., Griem, P., McNamee, P. M., Ryan, C. A., and Safford, R. J. 2008. Dermal sensitization quantitative risk assessment (QRA) for fragrance ingredients. *Regul. Toxicol. Pharmacol.* 52, 3–23.

Berenbaum, M. C. 1985. The expected effect of a combination of agents: The general solution. *J. Theor. Biol.* 114, 413–431.

Bonefeld, C. M., Nielsen, M. M., Rubin, I. M., Vennegaard, M. T., Dabelsteen, S., Gimenez-Arnau, E., Lepoittevin, J. P., Geisler, C., and Johansen, J. D. 2011. Enhanced sensitization and elicitation responses caused by mixtures of common fragrance allergens. *Contact Derm.* 65, 336–342.

Bosgra, S., van Eijkeren, J. C., van der Schans, M. J., Langenberg, J. P., and Slob, W. 2009. Toxicodynamic analysis of the combined cholinesterase inhibition by paraoxon and methamidophos in human whole blood. *Toxicol. Appl. Pharmacol.* 236, 9–15.

Cumberbatch, M., Scott, R.C., Basketter, D.A., Scholes, E.W., Hilton, J., Dearman, R.J., and Kimber, I. 1993. Influence of sodium lauryl sulphate on 2,4-dinitrochlorobenzene-induced lymph node activation. *Toxicology* 77, 181–191.

De Jong, W. H., Tentij, M., Spiekstra, S. W., Vandebriel, R. J., and Van Loveren, H. 2002. Determination of the sensitising activity of the rubber contact sensitizers TMTD, ZDMC, MBT and DEA in a modified local lymph node assay and the effect of sodium dodecyl sulfate pretreatment on local lymph node responses. *Toxicology* 176, 123–134.

Esser, P. R., Wolfle, U., Durr, C., von Loewenich, F. D., Schempp, C. M., Freudenberg, M. A., Jakob, T., and Martin, S. F. 2012. Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PLoS One* 7, e41340.

Gerberick, G. F., Robinson, M. K., Ryan, C. A., Dearman, R. J., Kimber, I., Basketter, D. A., Wright, Z., and Marks, J. G. 2001. Contact allergenic potency: Correlation of human and local lymph node assay data. *Am. J. Contact Derm.* 12, 156–161.

Hall, B., Steiling, W., Safford, B., Coroama, M., Tozer, S., Firmani, C., McNamara, C., and Gibney, M. 2011. European consumer exposure to cosmetic products, a framework for conducting population exposure assessments Part 2. *Food Chem. Toxicol.* 49, 408–422.

Jowsey, I. R., Basketter, D. A., and Irwin, A. 2008. Proliferative responses in the local lymph node assay associated with concomitant exposure to 1,4-phenylenediamine and methyl-2-bromo glutaronitrile: Evidence for synergy? *Contact Derm.* 59, 90–95.

- Kimber, I., Basketter, D. A., Gerberick, G. F., and Dearman, R. J. 2002. Allergic contact dermatitis. *Int. Immunopharmacol.* **2**, 201–211.
- Kimber, I., Hilton, J., Botham, P. A., Basketter, D. A., Scholes, E. W., Miller, K., Robbins, M. C., Harrison, P. T., Gray, T. J., and Waite, S. J. 1991. The murine local lymph node assay: Results of an inter-laboratory trial. *Toxicol. Lett.* **55**, 203–213.
- Martin, S. F., Esser, P. R., Weber, F. C., Jakob, T., Freudenberg, M. A., Schmidt, M., and Goebeler, M. 2011. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* **66**, 1152–1163.
- Moerbeek, M., Piersma, A. H., and Slob, W. 2004. A comparison of three methods for calculating confidence intervals for the benchmark dose. *Risk Anal.* **24**, 31–40.
- Natsch, A. 2010. The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers—functional relevance and a hypothesis on innate reactions to skin sensitizers. *Toxicol. Sci.* **113**, 284–292.
- Rastogi, S. C., and Johansen, J. D. 2007. Survey and risk assessment of chemical substances in deodorants. Danish Environmental Protection Agency. Survey of Chemical Substances in Consumer Products, No. 86.
- Slob, W. 2002. Dose-response modeling of continuous endpoints. *Toxicol. Sci.* **66**, 298–312.
- Slob, W., and Setzer, R. W. 2014. Shape and steepness of toxicological dose-response relationships of continuous endpoints. *Crit. Rev. Toxicol.* **44**, 270–297.
- Smith, H. R., Basketter, D. A., and McFadden, J. P. 2002. Irritant dermatitis, irritancy and its role in allergic contact dermatitis. *Clin. Exp. Dermatol.* **27**, 138–146.
- Uter, W., Goncalo, M., Yazar, K., Kratz, E. M., Mildau, G., and Liden, C. 2014a. Coupled exposure to ingredients of cosmetic products: III. Ultraviolet filters. *Contact Derm.* **71**, 162–169.
- Uter, W., Yazar, K., Kratz, E. M., Mildau, G., and Liden, C. 2013. Coupled exposure to ingredients of cosmetic products: I. Fragrances. *Contact Derm.* **69**, 335–341.
- Uter, W., Yazar, K., Kratz, E. M., Mildau, G., and Liden, C. 2014b. Coupled exposure to ingredients of cosmetic products: II. Preservatives. *Contact Derm.* **70**, 219–226.
- van der Veen, J. W., Gremmer, E. R., Vermeulen, J. P., van Loveren, H., and Ezendam, J. 2013. Induction of skin sensitization is augmented in Nrf2-deficient mice. *Arch. Toxicol.* **87**, 763–766.
- Wright, Z. M., Basketter, P. A., Blaikie, L., Cooper, K. J., Warbrick, E. V., Dearman, R. J., and Kimber, I. 2001. Vehicle effects on skin sensitizing potency of four chemicals: Assessment using the local lymph node assay. *Int. J. Cosmet. Sci.* **23**, 75–83.

## SUPPLEMENT

### Description of dose-response analysis using PROAST.

The analysis of both the range-finding study and the mixture experiment was performed with the PROAST software. The dose addition modelling was first implemented in version 50.9. Investigators might use the same software for experiments similar to these two experiments, as illustrated in this supplement. The PROAST software is available as an R-package ([www.proast.nl](http://www.proast.nl)), that is, it can be run after installing R ([cran.r-project.org](http://cran.r-project.org)) on the computer.

#### *Dose-range finding experiment*

For the analysis of the range-finding study the data are put in a format as illustrated in Supplementary Table 1 (data shown for the lowest dose groups only). The relevant chemical is indicated as a label in each row of a column called “chemical” (it is recommended to use short labels, as they are used in the legend of the plot). For the controls, the label is not relevant; here, they were entered evenly.

**Supplementary Table 1. Data format for the dose-range finding study.**

| dose | IFNg.per.ml | IL10.per.ml | chemical |
|------|-------------|-------------|----------|
| 0    | 174.197     | 272.3       | ie       |
| 0    | 263.888     | 242.65      | ie       |
| 0    | 109.395     | 263.11      | ie       |
| 0    | 223.217     | 294.88      | ie       |
| 0    | 248.689     | 157.38      | ca       |
| 0    | 125.135     | 221.12      | ca       |
| 0    | 202.441     | 128.19      | ca       |
| 0    | 99.228      | NA          | ca       |
| 1    | 201.458     | 116.39      | ie       |
| 1    | 262.708     | 239.64      | ie       |
| 1    | 183.847     | 127.09      | ie       |
| 1    | 586.494     | 225.05      | ie       |
| 2    | 442.162     | 195.33      | ie       |
| 2    | 413.5       | 257         | ie       |
| 2    | 293.921     | 190.62      | ie       |
| 2    | 163.182     | 162.64      | ie       |

After having saved the table in text format (tab delimited), open R, and load the PROAST package.

Then type

```
> data.pr <- read.table(data.txt, header = T)
```

where data.txt is the name of the saved table in text format and data.pr the data that is now created in PROAST format.

Now, the analysis can start by typing

```
> f.proast(data.pr)
```

which will evoke a number of questions that may be answered as indicated in Supplementary Table 2.

Note that these answers are as usual, the key question is the one asking for the model, which should be answered by 46 (denoting the four-parameter exponential model, including a BMD for the reference chemical, and an RPF for the other chemical).

**Supplementary Table 2. Illustration of a PROAST session fitting the exponential model including BMD and RPF as parameters.**

| question                                                               | answer | comment                                             |
|------------------------------------------------------------------------|--------|-----------------------------------------------------|
| What type of response data do you want to consider?                    | 1      | Individual continuous observations                  |
| Q1: Which variable do you want to consider as independent variable?    | 1      | dose                                                |
| Do you want to fit a nested set of models                              | 1      | Single model                                        |
| Q2: Which response variable do you want to analyse ?                   | 2      | IFNg per ALN                                        |
| Which model do you want to fit ?                                       | 46     | Exponential model in terms of RPF                   |
| give value for CES (negative for decreasing response)                  | 2      | = 200% or 3-fold increase                           |
| Give number of factor serving as covariate with respect to parameter b | 5      | Factor chemical                                     |
| Give number associated with the reference                              | 2      | ie = isoeugenol                                     |
| MAIN MENU: What do you want to do ?                                    | 4      | fit model                                           |
| Do you want to change the parameter constraints ?                      | 1      | no                                                  |
| give name for file to store results (or type 0 if none) >              | name   | e.g. IFNg.m46 (for the model number used)           |
| MAIN MENU: What do you want to do ?                                    | 5      | replot the data, to obtain the confidence intervals |
| Which plot type do you want ?                                          | 4      | Double log-scale                                    |
| Do you want color plot?                                                | 1      | yes                                                 |

|                                           |   |                                                                 |
|-------------------------------------------|---|-----------------------------------------------------------------|
| Do you want to plot the group means?      | 3 | group means only (to enable the option of confidence intervals) |
| Do you want confidence intervals in plot? | 2 | yes                                                             |

### *Mixture experiment*

The dose-addition analysis is also similar to a dose-response analysis of a usual dose-response dataset in PROAST, but the data need to have a specific format. The required data format is illustrated in Supplementary Table 3. The first two columns represent the doses applied for both chemicals. For marginal doses, the dose of the other chemical is zero, and for mixtures, both doses are nonzero. The first two letters in the names of the first two columns need to be chosen with care: they will be used as an indicator of the reference chemical in the output. Note that PROAST will use the first of the two columns as the reference chemical. The third column provides a label indicating which chemical was administered (or both). In particular, this label is needed to define the marks of the plotted data (see Fig. 3). It is recommended to use short labels (e.g. two letters), they are used in the legend with the plot, and only need to be recognizable by the analyst. Note that for the control group the label is only important for plotting, in this case the controls were equally often labelled with either chemical name. The next columns relate to the various parameters observed. The first row of the Supplementary Table 3 should provide names for each column; these names should not include spaces or symbols other than dots and underscores. A; other rows relate to a single observation (animal). Empty cells in the table are not allowed; if no value is available (such as missing values) enter NA in that cell.

**Supplementary Table 3. Data format of the mixture experiment**

| IE.dose | CA.dose | chemical | cells.ALN | IL10.ml | IFNg.ml          |
|---------|---------|----------|-----------|---------|------------------|
| 0       | 0       | IE       | 8029000   | 337     | 58               |
| 0       | 0       | IE       | 6675200   | 382     | 86               |
| 0       | 0       | IE       | 7413000   | 422     | 119              |
| 0       | 0       | IE       | 6443850   | 447     | 104              |
| 0       | 0       | CA       | 7171500   | 306     | 14 <sup>1)</sup> |
| 0       | 0       | CA       | 6647900   | 546     | 161              |
| 0       | 0       | CA       | 6144133   | 365     | 93               |
| 0       | 0       | CA       | 7710500   | 532     | 230              |
| 0.01    | 0       | IE       | 6905033   | 451     | 188              |
| 0.01    | 0       | IE       | 11172000  | 532     | 293              |
| 0.01    | 0       | IE       | 6534500   | 496     | 255              |
| 0.01    | 0       | IE       | 9646000   | 408     | 215              |

|      |      |     |          |      |      |
|------|------|-----|----------|------|------|
| 0    | 0.02 | CA  | 9789500  | 409  | 351  |
| 0    | 0.02 | CA  | 8564500  | 529  | 414  |
| 0    | 0.02 | CA  | 7042000  | 534  | 264  |
| 0    | 0.02 | CA  | 5351500  | 474  | 123  |
| 0.04 | 0    | IE  | 8036000  | 521  | 255  |
| 0.04 | 0    | IE  | 20458667 | 727  | 482  |
| 0.04 | 0    | IE  | 8596000  | 538  | 260  |
| 0.04 | 0    | IE  | 9068500  | 408  | 198  |
| 0    | 0.08 | CA  | 13863500 | 666  | 406  |
| 0    | 0.08 | CA  | 8354500  | 669  | 429  |
| 0    | 0.08 | CA  | 8169000  | 654  | 821  |
| 0    | 0.08 | CA  | 9831500  | 815  | 378  |
| 0.16 | 0    | IE  | 18224500 | 1721 | 1114 |
| 0.16 | 0    | IE  | 20618500 | 1945 | 1284 |
| 0.16 | 0    | IE  | 18798500 | 2081 | 1318 |
| 0.16 | 0    | IE  | 18375000 | 1405 | 1313 |
| 0    | 0.32 | CA  | 12453000 | 1028 | 2167 |
| 0    | 0.32 | CA  | 13804000 | 1013 | 1334 |
| 0    | 0.32 | CA  | 6221250  | 951  | 1282 |
| 0    | 0.32 | CA  | 12971000 | 938  | 1167 |
| 0.02 | 0.02 | mix | 8092000  | 714  | 574  |
| 0.02 | 0.02 | mix | 5680500  | 562  | 280  |
| 0.02 | 0.02 | mix | 7045500  | 800  | 577  |
| 0.02 | 0.02 | mix | 9464000  | 616  | 399  |
| 0.04 | 0.04 | mix | 7154000  | 950  | 684  |
| 0.04 | 0.04 | mix | 8876000  | 756  | 914  |
| 0.04 | 0.04 | mix | 10013500 | 1166 | 945  |
| 0.04 | 0.04 | mix | 9579500  | 713  | 354  |
| 0.04 | 0.12 | mix | 11000500 | 1049 | 1242 |
| 0.04 | 0.12 | mix | 11875500 | 845  | 1118 |
| 0.04 | 0.12 | mix | 9401000  | 884  | 856  |
| 0.04 | 0.12 | mix | 16597000 | 1393 | 1426 |
| 0.12 | 0.04 | mix | 15053500 | 1562 | 1570 |
| 0.12 | 0.04 | mix | 14206500 | 946  | 698  |
| 0.12 | 0.04 | mix | 13282500 | 993  | 527  |

|      |      |     |          |      |      |
|------|------|-----|----------|------|------|
| 0.12 | 0.04 | mix | 14941500 | 1559 | 1093 |
| 0.16 | 0.16 | mix | 14987000 | 1325 | 954  |
| 0.16 | 0.16 | mix | 16404500 | 1759 | 3026 |
| 0.16 | 0.16 | mix | 18112500 | 1400 | 1599 |
| 0.16 | 0.16 | mix | 18483500 | 1750 | 1745 |
| 0.24 | 0.08 | mix | 16961000 | 1932 | 3180 |
| 0.24 | 0.08 | mix | 14686000 | 1590 | 1816 |
| 0.24 | 0.08 | mix | 17199000 | 1431 | 1158 |
| 0.24 | 0.08 | mix | 13953333 | 1382 | 1811 |
| 0.08 | 0.24 | mix | 11791500 | 1228 | 1800 |
| 0.08 | 0.24 | mix | 17031000 | 969  | 1382 |
| 0.08 | 0.24 | mix | 13349000 | 982  | 965  |
| 0.08 | 0.24 | mix | 14703500 | 1089 | 1425 |

<sup>1)</sup> This value was detected as an outlier in the statistical analysis (Grubb test,  $P < 0.05$ ), and removed in the analysis as shown in Fig. 2A.

After having saved the table in text format (tab delimited), open R, and load the PROAST package.

Then type

```
> data.pr <- read.table(data.txt, header = T)
```

where data.txt is the name of the saved table in text format and data.pr the data in PROAST format.

Now, the analysis can start by typing

```
> f.proast(data.pr)
```

which will evoke a number of questions that may be answered as indicated in Supplementary Table 4.

Note that these answers are as usual, but the question asking for the independent variable (the dose) should now be answered by two numbers rather than one. In R, two numbers can be combined into one single answer by entering c(n1, n2), where n1 and n2 are the relevant columns numbers.

Another difference with an analysis of a non-mixture experiment is that the data need to be re-plotted after having indicated that the marks should depend on the labels in column “mix”.

**Supplementary Table 4.**

| question                                                            | answer | comment                                                                                          |
|---------------------------------------------------------------------|--------|--------------------------------------------------------------------------------------------------|
| What type of response data do you want to consider?                 | 1      | Individual continuous observations                                                               |
| Q1: Which variable do you want to consider as independent variable? | c(1,2) | Meaning : both 1 and 2, which are the first two columns, in this case the doses of each chemical |
| Do you want to fit a nested set of models                           | 1      | Single model                                                                                     |
| Q2: Which response variable do you want to analyse ?                | 4      | Cells.ALK                                                                                        |
| Which model do you want to fit ?                                    | 5      | Exponential model with four parameters                                                           |
| MAIN MENU: What do you want to do ?                                 | 4      | Fit model                                                                                        |
| Do you want to change the parameter constraints ?                   | 1      | no                                                                                               |
| give name for file to store results (or type 0 if none) >           | name   | e.g. cells.ALK.m5                                                                                |
| MAIN MENU: What do you want to do ?                                 | 1      | Change settings                                                                                  |
| Which setting(s) do you want to change?                             | 9      | Distinct plotting                                                                                |
| Q9: Which variable do you want to consider for distinct plotting?   | 3      | Column called mix                                                                                |
| Which setting(s) do you want to change?                             | 24     | continue                                                                                         |
| MAIN MENU: What do you want to do ?                                 | 5      | Replot the data, to obtain the confidence intervals                                              |
| Which plot type do you want ?                                       | 4      | Double log-scale                                                                                 |
| Do you want color plot?                                             | 1      | yes                                                                                              |

|                                           |   |                                                                 |
|-------------------------------------------|---|-----------------------------------------------------------------|
| Do you want to plot the group means?      | 3 | group means only (to enable the option of confidence intervals) |
| Do you want confidence intervals in plot? | 2 | yes                                                             |