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MUT/08/4

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)

DRAFT WORKING PAPER ON MUTAGENICITY ASSESSMENT OF CHEMICAL MIXTURES

Introduction

1. The COM expressed an interest in the evaluation of the mutagenicity of chemical mixtures during the 2005 and 2006 horizon scanning exercises. One recommendation from COM was to consider the possible occurrence of synergistic interactions regarding mutagenic effects of chemical mixtures, the possible mechanisms for any synergistic effects and the implications of such a finding for risk assessment. The COM evaluation outlined in this draft working paper is intended to build on the work of the COT Working Group on Risk Assessment of Mixtures of Pesticides and similar substances (WiGRAMP)¹ <http://www.food.gov.uk/science/ouradvisors/toxicity/cotwg/wigramp/> and the ongoing work of the Interdepartmental Group on Health Risks from Chemicals (IGHRC) on the risk assessment of chemical mixtures <http://www.food.gov.uk/science/ouradvisors/toxicity/cotwg/wigramp/>.² Thus the definitions and nomenclature used to describe interactions regarding mutagenicity induced by chemicals in this draft working paper have been taken from these reviews and are briefly commented on in paragraph 2 of this introduction.

2. The COT Working Group had noted that although there were a large number of studies on mixtures relatively few had appropriate data on the nature of the interactions between chemicals. The general principle reached from substantive consideration by the COT Working Group of data on pesticides across all toxicological end points was that in absence of data to the contrary, substances with similar modes of action could be assumed to act by dose-additivity, and substances with dissimilar modes of action could be assumed to act by effect additivity. The term interaction could imply a range of effects such as synergism, potentiation, supra-additivity, or sub-additivity. The COT working group had not specifically considered the most appropriate approaches to mutagenicity testing of mixtures or development of mutagenicity testing approaches to identify interactions with regard to mutagenicity.

Introduction to approaches to evaluation of mutagenicity of mixtures

3 A number of strategies have been considered for the evaluation of chemical mixtures.³ These include testing whole mixtures (integrative), fractionation of mixtures to determine mutagenic components (dissective, top-down approach), and investigations of interactions by testing simple combinations, recombined fractions, and spiking of mixtures/fractions (synthetic, bottom up approach). All of these approaches have been identified

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from literature searches with regard to mutagenicity testing, although relatively few studies of whole mixtures were identified. Approximately 110 research papers with potentially relevant information were identified for consideration during the COM review.

4. A discussion paper on the mutagenicity testing of whole mixtures, approaches to dissection (fractionation/concentration) of mixtures regarding mutagenicity, and the presentation of a draft strategy for mutagenicity evaluation of mixtures was considered at the February 2007 meeting.

<http://www.advisorybodies.doh.gov.uk/pdfs/mut0703.pdf>

5. A discussion paper which presented a systematic review of published literature (up to the beginning of June 2007) of studies which had examined the potential interaction between chemicals regarding mutagenicity was considered at the October 2007 meeting. The Committee also briefly discussed approaches to design and evaluation of synthetic studies investigating interaction between chemicals regarding genotoxicity. The COM considered the 'envelope of additivity' approach could be a useful approach to presenting data from studies designed to investigate potential interaction between chemicals with regard to mutagenicity and genotoxicity (outlined in paragraph 17 below). <http://www.advisorybodies.doh.gov.uk/pdfs/mut0715.pdf>

6. This draft working paper summarises the information contained in these discussion papers and the conclusions reached by COM.

Mutagenicity testing of whole mixtures, approaches to dissection (fractionation/concentration)

Whole mixtures

7. There were comparatively few studies where whole mixtures had been subjected to mutagenicity evaluation retrieved. An *in-vivo* approach to the mutagenicity testing of cooked meats was considered.⁴ The primary purpose for mutagenicity testing of whole mixtures outlined in the literature was the development of monitoring approaches to inform on risk reduction strategies. The studies need to be interpreted in terms of the overall mutagenic potency of the mixture and the sensitivity of the assay used to detect an effect, but it was noted that the data from such studies provided no information on the relative contribution of mutagenic chemicals present in the food or the interactions between chemicals regarding mutagenicity. A number of investigators have suggested that where there is evidence that components of a mixture do interact, and in particular where there is evidence of mutagenic synergy, then it might be prudent to evaluate whole mixtures as they exist to obtain appropriate information on mutagenic hazard.⁵ Anwar (1993) proposed the term 'total mutagenic burden' for whole mixtures⁶ However the failure to detect mutagenicity when complex mixtures (e.g. fried foods) or fractions (e.g. catalytically cracked clarified oil) are tested either *in-vitro* or *in-vivo* did not prove the absence of potentially mutagenic compounds.^{7,8} The COM agreed that testing whole mixtures first using an *in*

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vitro screen (such as the Ames test or SOS chromostest) would have the advantage of picking up evidence for potential interactions, such as synergy that could be missed by testing individual fractions or chemicals isolated from a mixture.

Approaches to dissection (fractionation/concentration)

8. The key elements to approaches that might be potentially used are shown below in figure 1;

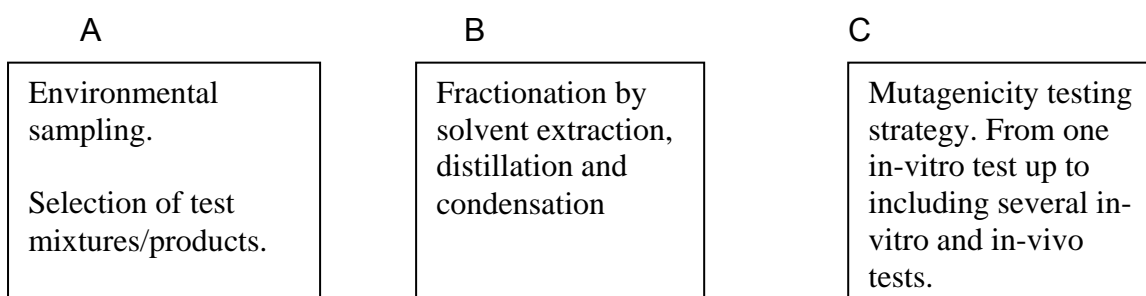


Figure 1; outline approach which could be used to evaluation of mutagenicity of chemical mixtures.

9. The COM considered published studies on the approach outlined in figure 1.⁹⁻²⁶ The COM agreed that a detailed review of environmental sampling for mutagenicity evaluation of mixtures was beyond the scope of the COM review. There were a wide range of factors which might affect the chemical mixture in samples recovered for mutagenicity testing including those affecting the emission of mixtures to the environment including variation in sources of release, distribution and degradation in the environment, the sampling procedure used (e.g. mass and volume of sample collected, the size distribution of particles in samples, the potential for reaction of sample with adsorbents/filters used in collection), and storage of samples prior to mutagenicity testing.⁹ Overall, it was concluded sampling strategies can significantly influence the estimation of mutagenicity of chemical mixtures and there is thus a need for the need for careful case-by-case approach to a sampling strategy with consistency of sampling procedure attained in order to generate mutagenicity data that are comparable.

10. The COM reviewed fractionation procedures using solvent extraction, distillation and condensation for a number of mixtures samples (diesel particles collected occupationally or environmentally^{13,14,15} or directly from exhausts^{20,22} or from fumes (e.g coke oven, roofing tar)¹⁷, oil based liquids^{16,23}, condensates or particles from pyrotechnic mixtures (e.g cigarette smoke^{9,12,19} or mixtures of known compounds¹⁹), hazardous wastes including industrial process effluents and municipal sludges¹⁸ and water samples taken from various points in the distribution system²¹). Most approaches used a single step extraction procedure. One particular difficulty in developing a strategy was optimising mutagenic response whilst avoiding excessive toxicity

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to the mutagenicity test indicator organisms used (e.g bacteria). Multi step procedures can result in loss or modification of mutagenic components. In an WHO sponsored International Programme on Chemical Safety (IPCS) led collaborative study of the mutagenicity of mixtures (urban air samples, diesel particles and coal tar solution) significant interlaboratory and intralaboratory variance in the results of *Salmonella typhimurium* TA98 and TA100 with or without exogenous metabolic activation was noted, which was partly due to the method of extraction (either soxhlet or ultrasonication) using dichloromethane as a solvent as well as the mutagenicity test procedures used.^{10,11} The final step in the fractionation procedure usually involved evaporation of extracts and resuspension in a solvent (usually DMSO) which is compatible with cell cultures used in mutagenicity tests and in-vivo mutagenicity test systems. This final step may also introduce a potential source of variation regarding mutagenicity test data.

11. The COM considered that general guidance could not be provided regarding fractionation procedures, and that the testing strategy would need to be considered on a case by case basis. Both the top down and bottom up approaches to mutagenicity testing of mixtures were considered to have potential applications in different circumstances.

12. The primary objective of the mutagenicity testing strategy for chemical mixtures should be to identify hazard in the tested material or mixture. A comparison of the mutagenicity test data for test mixtures derived from the same sources and subject to the same extraction and fractionation procedures may provide information for monitoring hazard of environmental samples, commercial products, pyrolysis products and hazardous wastes. The IPCS collaborative study also reported considerable variance with regard to strain of *Salmonella* used, the activation conditions and between replicate mutagenicity tests within the same laboratory.^{11,12} It is therefore likely that any successful approach to monitoring mutagenic hazard in chemical mixtures over a period of time would use well established sampling, extraction and fractionation procedures and mutagenicity testing procedures with a high degree of quality control for each step. Additional procedures could include spiking mixtures with compounds of known structure and mutagenic potential to investigate to investigate procedures used (e.g. extraction¹⁴ or pyrolysis¹²). Most studies are conducted to monitor chemical mixtures but it is possible to use an investigative approach regarding potential sources of mutagen release (e.g. the effect of agricultural run off on mutagenicity of water samples by timing and positioning sample collection from water courses²¹).

13. The majority of mutagenicity studies of chemical mixtures identified for the COM review used *Salmonella typhimurium* test strains as the only mutagenicity test.^{3,9,13,14,18,20,23,25} These studies may include exogenous metabolic activation systems selected to increase the number of revertant colonies formed for a particular tested mixture or to test for the mutagenicity of particular groups of compounds within a mixture (e.g. use of hamster S-9)²³ or selection of particular *Salmonella* strains (e.g. use of nitroreductase (NR)deficient strains²⁰, and NR and O-acetyltransferase deficient strains²⁵) or

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treatments (use of ROS scavengers such as α -tocopherol and/or ascorbate¹⁵) to monitor the mutagenicity of particular groups of mutagenic chemicals within the mixture. Additional *in-vitro* tests (e.g. using mammalian cells) can extend the potential for monitoring mutagenic hazard over a wider range of chemicals present in the mixture.

14. Relatively fewer studies use additional *in-vitro* and *in-vivo* tests.¹⁵⁻¹⁷ *In-vivo* mutagenicity tests are usually incorporated into testing strategies for single chemicals to confirm the potential for an compound of unknown mutagenic potential to induce effects *in-vivo*. The COM agreed that the inclusion of *in vivo* tests would have a confirmatory role only for monitoring of chemical mixtures, rather than being used routinely. This would be the case particularly when the environmental monitoring procedures concerned mixtures containing known *in vivo* mutagens, but possibly at levels below the level of detection in *in vivo* assays. One potentially useful approach inclusion of *in-vivo* tests in a strategy for monitoring complex mixtures was provided by Williams and Lewtas 1985¹⁷ who correlated the mutagenic response (slope of dose-response) to organic extracts from diesel, coke oven, roofing tar and cigarette smoke emissions from *in-vitro* tests (*Salmonella typhimurium* TA98 +S-9 (rat or hamster), and mouse lymphoma mutagenicity) with response in mouse skin tumour initiation assays. Having correlated mutagenic potency *in-vitro* and *in-vivo* (in this case between different mixtures) it would therefore be possible to continue monitoring of the comparative ranking of these mixtures with an *in-vitro* mutagenicity test strategy. It is possible to reach this conclusion as there was relatively good knowledge of the chemical composition of the mixtures included in the study, and a key hypothesis under test would have been the investigation of mixtures of PAHs which helped to define the *in-vitro* and *in-vivo* parts of the testing strategy.

15. The COM agreed an outline proposal for a strategy for monitoring mutagenicity of chemical mixtures (in particular occupational and environmental mixtures such as described in paragraph 10 of this draft working paper) using proposals for evaluating the mutagenicity of mixtures in the published literature^{24,26} but noted this was only general guidance and a case-by case approach was needed.

Preliminary considerations

- A. Collect information on chemical composition, and mutagenicity of chemicals in mixture. Define purpose of monitoring approach (is this to monitor overall mutagenic hazard, or to monitor selected levels of chemicals or groups of chemicals).
- B. Review literature for appropriate sampling, extraction and testing of similar mixtures. Review mutagenicity test data on specific chemicals in mixture or chemicals selected for monitoring.

With regard to mutagenicity testing

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- C. Define in-vitro testing strategy with focus on optimising and standardising the approach.
- D. Undertaken in-vitro monitoring to validate approach and identify sources of variance and their impact.
- E. Consider if necessary to develop an in-vivo segment to strategy. (For known in-vivo mutagens in a mixture, the default is to assume no threshold for mutagenicity in-vivo.)

Review of strategy

- F. Implement strategy and use data to inform on risk reduction strategies. Review strategy or add additional fractionation and identification of chemicals in monitoring scheme, only if a defined need is identified (e.g. sudden change in results of monitoring data).

Approaches to evaluating mutagenic interaction between chemicals

16. The design of synthetic studies to investigate the potential for interaction between chemicals, fractions or after spiking mixtures with chemicals is particularly complex. A number of factors to include, illustrated in the studies identified for review (for example^{3,12,25}) included the need for consideration of expected patterns of mutagenic response in bacterial tester strains used, the design of a testing strategy to limit the number of combinations tested to a minimum required to evaluate the nature of any interactions in mutagenicity tests (by selecting concentrations of test materials taking into account the dose-response of individual compounds or fractions in the tester strains, the consideration of the need for replicate experiments), and the consideration of the most appropriate approach to statistical analysis of data. The data could be analysed by a number of methods including the projections to latent structures (PLS) approach which overcomes many of the problems inherent in inter-correlated (dependent) predictor variables and produces results which are easily viewed.¹³

17. The COM agreed the concept of the envelope of additivity was potentially a helpful approach to graphically presenting the results of studies and to help identify non-interaction (e.g. dose-response and effect additive responses) and interaction responses (e.g. synergy and antagonism)²⁷ The COM noted the proposed unifying approach for application of statistical methods in chemical mixture research based on the shape of the dose response curve and changes in the slope of the dose-response in studies using two or more chemicals.²⁸ The approach suggested by Gennings et al linked the traditional statistical models of interaction (as found in the general linear model / factorial ANOVA models) to the different concepts of joint toxic action. The unification of the approaches is achieved by showing that there is no interaction if the dose-response relationship of one chemical is not changed by the presence of other chemicals. An interaction exists if there is a

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change in the slope of the response. This concept of interaction related to underlying statistical models of additivity. Members agreed that the approach suggested by Gennings et al 2005 could be potentially helpful when assessing mutagenicity studies of interaction between chemicals.

Review of studies investigating the potential interaction between chemicals regarding mutagenicity

18. A total of 91 research papers were identified by literature searches up to June 2007. A quality scoring approach was used to select the best quality studies for further review by COM. The quality screening approach was based on Borgert et al 2001²⁹ for evaluating interaction studies in terms of the quality of design, data and interpretations. Reliable interaction studies were considered to be those that are interpretable without making assumptions about untested and unanalysed parameters. (An overview of the quality scoring criteria is given in Annex 1 to this draft working paper.) Very few ($n=15$) published studies met all five of the criteria and these were considered in detail.³⁰⁻⁴⁴ Brief summaries of other papers not meeting all of the quality screening criteria were also provided for the COM.

19. The COM agreed that the well-conducted studies of defined mixtures of mutagenic chemicals did not provide a consistent picture of combination effects being predictable on the basis of the single agent dose-response information. In the majority of cases, substances tested in these studies are mutagens with relatively well understood mechanisms of action (e.g. B[a]P, and the alkylating agents EMS, MMS, MNU). In only one instance was the same combination of chemicals tested (EMS and ENU) in two different tests (Ames³⁶ and in an *in vivo* mouse micronucleus test⁴²). Kawazoe and colleagues showed that in the Ames assay EMS and ENU induced linear dose-responses and that using dose addition it was possible to model the combined effect of these chemicals.³⁶ In the mouse micronucleus assay, these chemicals induced non-linear dose response curves, but mixture effects were additive when compared to dose addition predictions.⁴² For other combinations of alkylating agents, however, it is not clear why additivity is not observed. In many of these cases, observed mixture effects appear to fall within the additivity envelope and as some investigators do not estimate confidence 'belts' for the additivity predictions, it is possible the observations are not truly statistically significantly different from the non-interaction predictions.

20. The COM considered that an important part of the assessment of genotoxicity studies of interaction between chemicals would be reproducing results seen in one test system with other appropriate genotoxicity tests (e.g. confirming results seen in bacterial gene mutation assays in mammalian cell gene mutation assays). This could be used in a weight of evidence assessment of interactions and would be particularly important for assessment of interactive effects such as synergy or antagonism. The strategy for assessment of interaction with regard to mutagenicity would also need to include *in vivo* tests with appropriate consideration of toxicokinetics

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and exposure of sampled tissues. Members commented that the available published literature presented a number of examples^{35,37,38,40} where interaction had been reported, but there was essentially no appropriate independent confirmation of the results in separate tests, or within an appropriate mutagenicity testing strategy for the identification of interactions and no definite conclusions could be reached.

21. The COM considered the four available published studies which reported the best evidence for interaction in detail to provide advice on possible mechanisms of mutagenicity might be associated with interaction.

22. Homme M et al (2000)³⁵ had documented synergistic DNA damage using UDS assays in human fibroblasts between 4-nitroquinoline-1-oxide (4-NQO) and non-effective methyl methanesulfonate (MMS). The authors had proposed that the ultimate DNA reactive metabolites formed from 4-NQO resulted in unwinding of super helical DNA so that more molecules of MMS could reach the bases of DNA resulting increased methylation and mutation. The COM considered that a viable hypothesis had been proposed. It would be necessary to undertake independent confirmation of the results and to include additional combinations of mutagens with and without 4-NQO to provide further data to investigate the proposed mechanism. At present no definite conclusions could be reached on this specific example of an interaction.

23. Kojima H et al (1992)³⁷ had investigated the potential for interaction between MMS and EMS in Chinese hamster V79 cells using cell killing, induction of 6-thioguanine mutants (6TG resistant mutants) and chromosome aberrations. These authors had reported evidence for synergistic interactions for both cell killing and 6TG mutation and evidence for additivity with regard to chromosome aberrations. The authors had suggested that the DNA damage produced by one alkylating agent could be increased in the presence of a small amount of another alkylating agent. The COM noted the predominant SN₂ mechanism of MMS and the SN₁ mechanism of EMS and considered that these differences could form the basis for a hypothesis of interactive effects with regard to genotoxicity. However the COM considered there was a need for independent confirmation of these results and further investigations of other alkylating agents before any definite conclusions could be reached.

24. Lutz WK et al (2005)³⁸ had reported evidence for antagonism using a combination of N-methyl-N-nitrosourea (MNU) and the topoisomerase-II inhibitor genistein (GEN) in the mouse lymphoma assay in LY5178Y cells. In separate tests when MMS was combined with GEN an additive response (reported to be within the envelope of additivity) was reported. The authors hypothesised that the profile of DNA methylation and or epigenetic effects were responsible for the different responses reported for the binary combinations tested. The COM considered these investigations raised interesting hypotheses for further testing but no definite conclusions could be reached on these data.

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25. Marrazzini A et al (1994)⁴⁰ had undertaken *in vivo* mouse bone marrow MN tests in mice using intraperitoneal administration of binary combinations of hydroquinone, catechol and phenol. Mixtures of hydroquinone and phenol and catechol and phenol were reported to result in synergistic induction of micronuclei. Members noted that it was not possible to discern a potential mechanism of interaction from these studies which could be used to support hypotheses for further testing.

26. The COM was aware of the different interpretations of the term synergy was used by the research groups and the limitations in the available data made it difficult to reach any definite conclusions. However, overall there was insufficient evidence to conclude that the studies reviewed provided conclusive evidence for interaction effects (either synergy or antagonism). However, a number of the studies provided evidence to suggest hypotheses for interaction (see paragraphs 22-25 above) which could be further examined in appropriately designed mutagenicity testing strategies. The COM agreed that the potential for interactions between chemicals with regard to genotoxicity needed to be studied on a case-by-case basis.

COM Discussion and Conclusions

Whole mixtures

27. The COM considered mutagenicity testing of whole mixtures, approaches to dissection (fractionation/concentration) of mixtures. The primary purpose of such studies would be to monitor mutagenic response in tests for a wide variety of mixtures (for example foods, samples of pollution (air and water) condensates or particles from pyrotechnic mixtures (e.g. cigarette smoke or mixtures of known compounds), hazardous wastes including industrial process effluents and municipal sludges. The COM noted there was comparatively little data on mutagenicity testing of whole mixtures available. The COM agreed that testing whole mixtures first using an *in vitro* screen (such as the Ames test or SOS chromotest) would have the advantage of picking up evidence for potential interactions, such as synergy that could be missed by testing individual fractions. However the failure to detect mutagenicity when complex mixtures (e.g. fried foods) or fractions (e.g. catalytically cracked clarified oil) are tested either *in-vitro* or *in-vivo* did not prove the absence of potentially mutagenic compounds.

Approach to dissection of mixtures

28. The COM agreed an outline proposal for a strategy for fractionation and monitoring of mutagenicity of chemical mixtures (as outlined in para 10 and 27 of this draft working paper) but noted this was only general guidance and a case-by case approach was needed.

Approach to evaluation of studies to investigate interactions

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29. The COM agreed the concept of the envelope of additivity was considered to be a helpful approach to graphically presenting the results of studies and to help identify non-interaction (e.g. dose-response and effect additive responses) and interaction responses (e.g. synergy and antagonism). The COM noted the proposed unifying approach by Genning and colleagues (see reference 28 in this draft working paper) for application of statistical methods in chemical mixture research based on the shape of the dose response curve and changes in the slope of the dose-response in studies using two or more chemicals and agreed this could be of potential use in evaluating genotoxicity.

Review of published studies on interaction between chemicals with regard to genotoxicity.

30. The COM noted that the available published literature presented a number of examples^{35,37,38,40} where interaction with regard to mutagenicity had been reported, but there was essentially no appropriate independent confirmation of the results in separate tests, or within an appropriate genotoxicity testing strategy for the identification of interactions and no definite conclusions could be reached.

31. The COM agreed that the available studies had raised a number of potential hypotheses regarding interaction between ultimate DNA reactive chemicals and DNA structure, (e.g. different mechanisms of DNA alkylation), the effect of covalent binding to DNA of one chemical on the potential for other reactive metabolites and chemicals to bind to DNA and possible epigenetic mechanisms which could potentially result in a mutagenic response that resulted from an interactive effect between chemicals (i.e. synergistic or antagonistic). There was a need for further research regarding such mechanisms, which if confirmed in an appropriate mutagenicity testing strategy might be of potential significance for public health.

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ANNEX1 TO DRAFT WORKING PAPER ON MUTAGENICITY EVALAUTION OF MIXUTRES.

APPROACH TO QUALITY SCREENING OF PUBLISHED PAPERS ON INTERACTION STUDIES: SUMMARISED FROM BOERGERT CJ ET AL (2001) HUM ECOL RISK ASSESS, 7, 259-306.

1. In 2001, Borgert and colleagues (*Hum Ecol Risk Assess* 7(2): 259-306, 2001) proposed a set of criteria for evaluating interaction studies in terms of the quality of design, data and interpretations. Reliable interaction studies are those that are interpretable without making assumptions about untested and unanalysed parameters. Although there is debate among experts regarding which models of non-interaction, which methods of combination analysis, and which statistical tests are most appropriate, it was still possible to apply the principles outlined by Bogert et al to assist in data interpretation. The criteria proposed were designed to assist risk assessors in identifying studies that can be used in component-based mixture risk assessments as well as those studies that are less useful due to inadequacies in design or interpretation. The aim was for them to apply broadly to interaction data for all effects of drugs, pesticides, industrial chemicals, food additives and natural products.

2. These criteria appear to provide a useful basis on which to evaluate the studies identified on mutagenic interactions. The five criteria set out below have been refined where necessary to facilitate their specific application to genetic toxicology studies and then used to evaluate the 91 retrieved articles.

I. Dose-response relationship for the individual mixture components are adequately characterised

Without adequate dose-response relationship characterisation for the individual components, it is not possible to determine whether a biological effect of a mixture is due to interactions between the components.

Ideally, single agent dose-response characterisation should enable slope, inflection points, and maximum and minimum effects to be estimated. Most importantly, key to being able to decide the appropriate 'no interaction' hypothesis (Criterion II, below) is whether the individual components of the mixtures have linear or non-linear dose-response curves and whether they have similar slopes. Inadequate characterisation of the dose-response relationship can lead to erroneous conclusions of interactions and this might be compounded further if the mixture components have significantly different shaped dose-response relationships.

For the purposes of this COM review, it was decided to focus, in the first instance, on mixtures of chemicals where all components are mutagenic. That is, evidence of "potentiation" from mixtures of mutagens with co-mutagens has not been considered at this point. Therefore, it is assumed that each mixture component alone induces a measurable genotoxic effect and detailed dose-response data are available.

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II. An appropriate ‘non-interaction’ or ‘additivity’ hypothesis should be, a priori, explicitly stated and used as the basis for assessing combination effects.

Interactions are inferred when a mixture of chemicals produces a biological response greater or less than expected based on mathematical concepts of additivity (non-interaction). Two models of non-interaction have been well-developed in the pharmacological and toxicological literature and are appropriate as the basis for non-interaction hypotheses. Dose addition is based on the concept that an agent cannot interact with itself, and predicts that two non-interacting compounds will behave as dilutions of one another when combined. The second model is response addition, and expresses probabilistic independence between two compounds. In this case, independence implies functional independence between two chemicals such that the incremental effect of one compound is unchanged in the presence of a second.

In the literature, dose addition largely assumes a strictly similar mechanism of action of all mixture components, while response addition is based on the idea of completely dissimilar mechanisms of action of the mixture components. Therefore, if mechanisms of action are well-enough understood, this may suggest the most appropriate non-interaction model to assume. However, in most cases adequately detailed understanding of the toxicological mechanisms of action for the individual mixture components is not available. Therefore it may be useful to compare observed combination responses with both models of non-interaction. In so doing, applying both models will generate a range of effects delineated by dose addition and response addition, referred to by some researchers as an ‘additivity envelope’, in which a non-interacting mixture would be expected to lie (**Figure 1**). This approach would be considered to meet this criterion. In addition, as the number of individual components in the mixtures of interest increases, it is likely that there will be a variety of chemicals with similar and dissimilar mechanisms of action and it may not be appropriate to use dose addition or response addition. In this regard, some groups are beginning to combine the two models, but as an interim, it is feasible to assume effects will lie in the additivity envelope if the mixture is non-interactive.

It should be noted, that dependent on the default non-interaction model applied, there are different demands made on the *ideal* single substance dose-response data (which has an impact on Criterion I). That is, for dose addition, single substance studies have to provide concentration-effect data for the same effect levels that will be assessed in the combination studies. For the application of response addition, it is necessary to have detailed resolution of the single substance dose-response relationships at effect levels below the region of interest for the mixtures.

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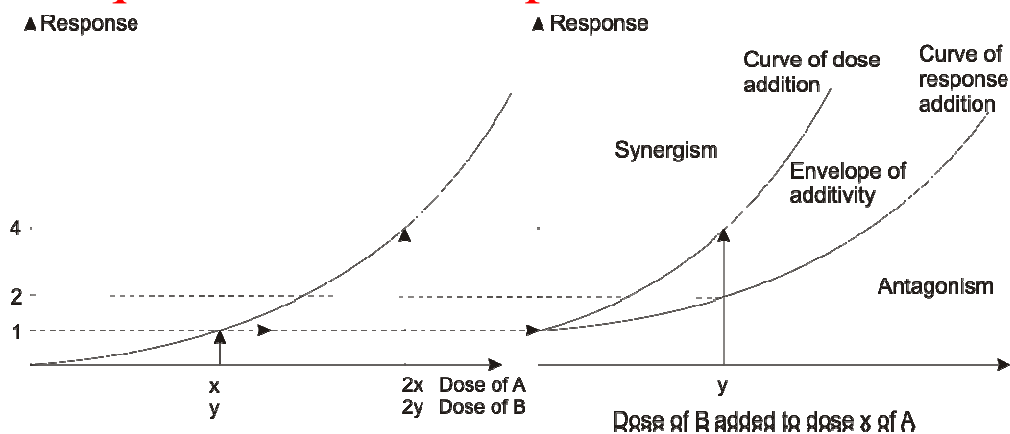


Figure 1. Schematic representation of a non-linear dose-response relationship (left hand side for two substances, A and B) and classification possibilities for the response of a mixture of the two components (right hand side; dose response for B added to dose x of A). Taken from Lutz *et al.* (2005). Dose x of chemical A produces a response of 1 effect unit, and dose y of chemical B has the same effect magnitude, in fact chemicals A and B have the same dose-response curves. A mixture of dose x of substance A plus dose y of substance B generated a response of effect level 4, one might postulate that A and B acted in a synergistic manner. This interpretation is not correct when the shape of the chemicals' dose-response curves are considered. Therefore, the mixture of dose x of substance A plus dose y of substance B can be considered as dose 2x of chemical A or 2y of chemical B, and these doses generate a response of effect level 4, i.e. in agreement with dose addition. If the two chemicals acted independently of each other, the expectation would be the lower of the two curves in the right hand panel, i.e. response addition. This curve has exactly the same shape as the dose-response on the left hand panel, except that it is set off on the y-axis by response level 1 (the effect generated by dose x of A). On this basis, the mixture of dose x of substance A plus dose y of substance B would result in effect level 2 as shown by the lower dotted line on the right hand panel.

III. Combinations of mixture components should be assessed across a sufficient range of concentrations and mixture ratios to support the goals of the study

The characteristics of a mixture are clearly dependent on the components of the mixture and the concentration range of the mixture that is tested. However, there may also be considerable dependence on the ratios at which each component is present within the mixture. This is because different types of interactions can be exhibited by the same mixture of chemicals at different mixture ratios. Approaches to mixture testing routinely used include:

- full factorial design: tests a full complement of component ratios across the dose-response range of each mixture ratio.
- fractional factorial design: reduces the number of tests to a specified subset of mixture combinations while still maintaining a substantial proportion of the information that would be produced with a full factorial design.
- ray design: tests fixed-ratio mixtures, i.e. a constant ratio of the mixture components, across a range of concentrations.

There are no hard and fast rules as to the correct approach to take in all cases, but it is important to employ the design that will satisfy the goals of the study, and not to over-interpret the resulting data. Detailed descriptions of these different approaches have been published recently (IGHRC, US EPA etc.)

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IV. Formal statistical tests should be used to determine whether the response produced by a combination is different from that predicted by the additive hypothesis.

Some researchers evaluate only whether responses differ statistically from controls and whether dose combination responses differ statistically from individual component responses. Such comparisons do not actually address the question of whether there is an interaction. As detailed in Criterion II, the appropriate non-interaction model will have been stated, and statistical tests should compare the observed mixture effect with that of the expected joint effect on the basis of the non-interaction hypothesis. Without a clearly stated non-interaction hypothesis, the results of any statistical test cannot be interpreted. Statistical methods that have been used to infer that mixture components interact include simple *t*-tests, linear models (including ANOVA and multiple regression) and multivariate regression. Ideally, the statistical approaches will allow confidence intervals to be placed on the observed mixture data and also on the predictions based on the mathematical models of dose addition or response addition. As the prediction is based on experimental (variable) data on the single substances, it is possible to estimate the variability associated with the predicted combined effect.

V. Interactions should be assessed at relevant levels of biological organisation.

Although the primary objective of the mutagenicity testing strategy for chemical mixtures should be to identify hazard in the tested material or mixtures, it is important to understand if the mixture poses a significantly greater hazard than the individual components. Identifying a potential interaction which might be of potential importance for public health, therefore requires not only a mechanistic rationale, *in vitro* evidence of interaction and *in vivo* evidence of interaction but also, the information must consistently point towards a synergistic interaction.

Interaction studies at the level of the whole organism or population can be difficult to interpret without information from underlying levels of biological organisation. Without knowledge of the mechanism of action of the mixture components it may not be possible to establish which non-interaction hypothesis is most appropriate. It may therefore be necessary to employ an additivity envelope approach (as detailed above in criterion II), consequently reducing the chance to detect true interactions. On the other hand, numerous interactions may be detected in studies carried out at the molecular, biochemical or cellular level, and these interactions may never manifest change in the organism.

Ideally the systems used to assess combination effects should be fit for purpose, which implies use of accepted mutagenicity/genotoxicity tests.

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