COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Statement on Food Standards Agency-funded research on health effects of mixtures of food additives (T01040/41)

Background

1. Traditionally risk assessment has been carried out on individual chemicals. However, this does not reflect the real-life situation as humans are seldom, if ever, exposed to single chemicals and all foods are mixtures of many different chemicals. Chemicals may exert combined effects related to either concomitant or sequential exposure, depending on their toxicokinetic and toxicological properties. In recent years concern about the possible "cocktail" effects of mixtures of chemicals, and in particular possible combination effects at low doses, has stimulated research. In a report in 2002 the COT made recommendations on approaches to risk assessment of mixtures of pesticides and similar substances[1,2], and in 2004 it considered whether these could be applied to mixtures of additives and contaminants[3] and outlined other approaches used for assessing mixtures of additives and contaminants. The COT also commented[4] on the Draft guidance document on "Chemical mixtures: a framework for assessing risks"[5] prepared by the Interdepartmental Group on Health Risks from Chemicals (IGHRC)

2. Table 1 shows the terminology used in describing the toxicology of mixtures.

3. In 2001 the Food Standards Agency commissioned a project (Research on health effects of mixtures of food additives**)** which was carried out jointly at Leatherhead Food International, Leatherhead, Surrey and TNO Quality of Life, Zeist, the Netherlands. As part of an Horizon Scanning exercise at its meeting on February 5th 2008, the COT was provided with a summary of the results of this project and agreed it would like to have an opportunity to comment on the full report. This was discussed by the committee at its meeting on April 1, 2008. To date this project has generated three publications[7, 8, 9] with at least two additional ones in preparation.

4. The project set out to build on existing information on the mode of action of a range of food additives compiled by the ILSI-Europe Acceptable Daily Intake Task Force[10, 11]. This had identified a number of additives where different types of combined action were plausible but it was not possible to predict which were more likely. Four additives that had been shown to cause liver enlargement were selected (Table 2).

Table 1: Terminology used in describing possible combined actions of chemicals in a mixture (based on COT 2002, after Cassee *et al.*[6])

Concept of type of combined behaviour	Terms used in this report	Synonyms	Observed effects
non-interaction - components of a mixture do not affect each other's toxic response.	simple similar action	simple joint action summation	Concentration/dose addition Chemicals have the same effect on the body and differ only in potency. The combined effect can be estimated from the total dose of all agents together, after adjusting for potency.
	simple dissimilar action	simple independent action independent joint action	Either response addition or effect addition. The modes of action and often the nature and site of effect differ among the chemicals in the mixture. "Response" reflects incidence data and response addition is determined by summing the incidence data for each component in the mixture. "Effect" reflects continuous data and effect addition is determined by summing the effect of each component in the mixture. Note that response and effect are sometimes used interchangeably.
interaction	potentiation	synergy supra-additivity	The combined effect of agents is greater than would be expected on the basis of dose-addition (if the chemicals have the same mode of action) or response-addition (if they do not have the same mode of action).
	antagonism	sub-additivity	The combined effect of agents is less than would be predicted by dose or effect/response addition

5. Table 2 shows the acceptable daily intakes (ADIs) set by the European Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA) for the four compounds, and the NOAELs identified by Groten *et al.*[10]. For all four compounds, these NOAELs were based on observations of liver enlargement associated with induction of hepatic enzymes at the next highest dose Data were collated from a series of different papers, not all of which measured the same enzymes. The ADIs set by the SCF are currently being reviewed by the European Food Safety Authority (EFSA), starting with the colours.

Outline of the study

6. Studies were carried out in the rat using dietary administration with individual food additives, binary mixtures of all six pairings of the four compounds and quaternary mixtures of all four compounds. In addition, *in vitro* studies were carried out with the individual additives and mixtures of additives in cultured rat and human hepatocytes, in order to provide a direct comparison of the effects in human and rat liver.

Table 2 – Additives used in the mixtures research

Additive	E Number	Uses	ADI (mg/kg bw)	NOAEL[10] (mg/kg bw/day)
Butylated hydroxytoluene (BHT)	E 321	Antioxidant	SCF: 0 -0.05 [12] JECFA: 0 -0.3 [13]	25
Propyl gallate	E 310	Antioxidant	SCF: 0 – 0.5 [14] JECFA: 0 -1.4 [15]	135
Curcumin	E 100	Colour	SCF: ADI not specified[16] JECFA: 0 -3 [17]	220
Thiabendazole as an additive	E 233	Previously used as fungicide mainly on a range of fruits. No longer permitted	SCF: was 0 - 0.3 [18] JECFA: 0 – 0.1 [19]	10
Thiabendazole as a pesticide and veterinary medicine	N/A	Pesticide and veterinary medicine	EC: 0.1[20] JECFA: 0.1[21]	10

In vivo studies

7. In preliminary range-finding studies, the food additives were fed individually to male Sprague-Dawley rats for 28 days to determine dietary levels of the compounds for use in the mixtures study and to identify biomarkers of effect for the individual compounds. Five concentrations (plus a zero control) were selected for each compound, based on data from the literature and historical data collated from 28 day studies in rats carried out in accordance with good laboratory practice (GLP). Dietary concentrations were selected to provide target doses of 25 to 1000 mg/kg bw/day for BHT and curcumin, 20 to 600 mg/kg bw/day for propyl gallate and 10 to 500 mg/kg bw/day for thiabendazole. The dietary concentrations ranged from 254 to 10154 mg/kg for BHT, from 254 to 10154 mg/kg for curcumin, from 203 to 6092 mg/kg for propyl gallate and from 102 to 5077 mg/kg for thiabendazole.

8. The highest dose level to be used for each of the compounds in the mixtures study was selected on the basis of the results of the endpoints examined in the preliminary study, including effects on body weight, liver weight and the various biomarkers measured (e.g. enzyme activities, mRNA levels). These highest (100%) dose levels were described by the researchers as being around or at the minimum observed adverse effect levels in the preliminary study, which differ from the NOAELs in Table 2 cited by Groten *et al.*[10]. Based on the dietary concentrations and food consumption, the achieved 100% doses were 333 mg/kg bw/day for BHT, 408 mg/kg bw/day for curcumin, 290 mg/kg bw/day for propyl gallate and 153 mg/kg bw/day for thiabendazole. The main mixtures study included 27 treatment groups and a control group, with each compound administered at combinations of 0, 25, 50 and 100% of the highest dose

9. The rationale for this protocol was to ensure that the 100% dose levels produced clear effects on some of the parameters measured, so that any effects due to combinations of chemicals in a mixture might be detected at lower individual dose levels (e.g. 25% of the total) when included in a mixture. The compounds were administered at 25, 50 and 100% of their maximum dose when given individually. For binary mixtures the individual compounds were each administered at 25 and 50% of

their maximum dose, such that the total fractional dose added up to 50 or 100%. For quaternary mixtures the individual compounds were each administered at 6.25, 12.5 and 25% of their maximum dose such that the total fractional dose was 25, 50 or 100%. This protocol was designed to test if the observed findings were most compatible with those predicted by effect addition, dose addition or interaction (synergy or antagonism) based on statistical analysis of the dose response relationship.

10. At the end of the 28 day dosing period the animals were killed, blood was sampled and livers removed. Clinical chemistry was carried out on the blood samples. The livers were divided to provide RNA samples for TaqMan® analysis, material for transcriptomics, subcellular fractions for measurement of enzyme activities and fixed samples for histological examination. Samples from animals of the same treatment group were pooled for transcriptomics.

11. Analysis of gene expression data on the effect of treatment with BHT, curcumin, propyl gallate and thiabendazole individually was carried out using a cDNA chip containing about 3000 different sequence verified rat cDNAs. Microarray analysis was carried out in accordance with the principles of Minimum Information About a Microarray Experiment (MIAME)[22]. Analysis of samples from the mixtures study was conducted using the Affimetrix GeneChip platform which provided a more robust system and a much larger number of probe sets (15923). Functional analysis of gene expression changes was performed using T-profiler[23], which is a TNO in-house (toxico)genomics database and analysis tool that allows comparison of systems toxicology/genomics datasets at the level of networks and pathways.

12. Predicted data for mixtures were derived from additivity surface equations obtained from response curve modelling for the individual additives. If measured data for mixtures were significantly different from predicted data, either on the assumption of dose or effect additivity, it was assumed the combined effects reflected interactions.

In vitro studies

13. Studies were performed to investigate the food additives and food additive mixtures in cultured rat and human hepatocytes using a 72 hour incubation period. Rat hepatocytes were treated with 40 concentrations of either individual food additives or mixtures of food additives. Chosen biomarkers of effect were CYP1A2 and CYP2B1 mRNA levels and 7-benzyloxy-4-trifluoromethylcoumarin (BFC) O-debenzylase activity. (BFC is a substrate for CYP1A2 and CYP2B1). The human hepatocyte study consisted of a control and 27 concentrations of either individual food additives or mixtures of food additives and CYP1A2, CYP2B6 and CYP3A4 mRNA levels as biomarkers of effect. CYP1A2 mRNA was therefore the only parameter measured in both rat and human hepatocytes.

Brief Summary of the Results

14. The authors concluded that, for body weight and liver weight, most of the findings were consistent with dose addition. In most cases liver weights showed no substantial deviations from predicted values either in binary or quarternary mixtures.

In the quarternary mixture with a total fractional concentration of 100% (25% of the maximum dose of each additive), the measured liver weight was 122% of control compared to the predicted value of 108% of control, indicating some deviation from the predicted value. However, as significant deviations from predicted values were not seen for liver weights in binary mixtures containing curcumin and propyl gallate, these results were considered by the authors to be primarily due to the relatively high dose levels of BHT and thiabendazole, both of which produced significant increases in relative liver weight without any evidence of hepatotoxicity.

15. The largest difference between predicted and measured values was for CYP1A2 expression, with all binary and quaternary mixtures showing antagonism. Apart from mixtures of BHT plus curcumin, this appeared to be a dose-dependent effect. Also, for all of the binary mixtures, at least one of the dose groups was not compatible with the concept of dose addition. Therefore, it is likely that antagonistic interactions occurred between these mixtures with respect to CYP1A2 mRNA expression.

16. Although curcumin alone did not induce the activity of glutathione S-transferase (GST), the induction observed with the combination of BHT and curcumin exceeded that seen with BHT alone, indicating an interaction. The induction was more pronounced for activity with 1,2-dichloro-4-nitrobenzene (DCNB), a marker of GSTmu forms, than with 1-dichloro-2,4-nitrobenzene (CDNB), which is a more general substrate for the different forms of GST.

17. Thiabendazole was found to have the most marked effects on the gene expression profile and also had a dominant effect in studies with binary and quaternary mixtures. Although curcumin and propyl gallate had only modest effects on gene expression and enzyme activity when administered individually, marked effects on gene expression were seen with binary combinations of these compounds.

18. In the studies conducted in rat and human hepatocytes, some quaternary mixtures produced antagonistic effects on CYP1A2 mRNA expression, as seen in the *in vivo* study. Both sub- and supra-additive deviations from predicted values were observed in the expression of CYP2B1 (rat), CYP3A4 (human) and CYP2B6 (human) with some binary and quaternary mixtures, but there was no consistent pattern across species or between the *in vitro* and *in vivo* studies.

19. In summary, no evidence of combination effects leading to overt toxicity was apparent with the conventional toxicological endpoints, whereas some interactions were observed for induction of GST activity and at the genomic level using transcriptomics.

Previous COT evaluations of relevance to mixtures

20. In 2004, the COT agreed general conclusions on mixtures of chemicals in food, extending the conclusions of its 2002 report[2] to take account of the possibility that exposure to some food additives and ingredients of very low toxicity may be much higher than exposure to pesticides and veterinary medicines"[3]. These conclusions were:

i. *“Because of the complexity and variability of chemical mixtures that may occur in the environment, risk assessment of any toxic effects of chemical mixtures is extremely difficult. Most experimental work has been directed at toxic effects due to combined actions on biological systems at relatively high levels of exposure in laboratory experiments in laboratory animals or using in vitro systems.*

ii. *Direct chemical reactions can occur between the components of a mixture: there are relatively few studies of these substances that have investigated such reactions.*

iii. *Several studies claim to have identified synergistic interactions of some mixtures. However, for the most part, these studies have been inadequately designed and based on an incomplete understanding of the concepts involved, but a few well-designed studies have demonstrated the occurrence of both synergistic and antagonistic interactions, as well as additive effects in mixtures. These effects have usually been demonstrated at high concentrations or high experimental exposure levels, which are probably unrepresentative of exposure doses to chemicals present at very low levels in food.*

iv. *Some interactions may not be easy to predict, such as those that may occur at the transcriptional level of the genome or second messenger signalling pathways.*

v. *The type of combined action or interaction found at clearly toxic effect levels may not predict what will happen at non-toxic levels, including levels only slightly lower than the lowest observed adverse effect levels (LOAELs).*

vi. *In relation to most examples of possible human exposure to multiple residues, it will be important critically to evaluate whether any effects are likely to occur at low levels of exposure, such as those that will occur through food and water.*

vii. *Studies in vivo with chemicals that exhibit the same mode of action in the same target organ have shown that the effects of mixtures of similarly acting toxicants show additivity (dose addition), which results from simple similar action. This is the case, over the whole dose range.*

viii. *It is essential to know what happens at non-toxic levels, including exposure levels just below the LOAEL, in order to assess the health risk for humans exposed to mixtures of pesticides, veterinary drugs and similar substances. Generally, when exposure levels of the chemicals within a mixture are in the range of the NOAELs, and the components of the mixture have different modes of toxic action, no additivity and no potentiating interactions are found, indicating the applicability of the basic concept of "simple dissimilar action”, which suggests that adverse reactions would be unlikely.*

ix. *Some studies (acute and subacute toxicity, genetic toxicity, carcinogenicity) have addressed the combined effect of mixtures of pesticides and in a few studies*

clear cases of potentiation were observed in animals exposed to levels of toxic substances showing adverse effects of individual compounds. However, direct extrapolation of these findings to much lower dose levels is not valid. Thus the probability of any health hazard due to additivity or potentiating interaction of mixtures of pesticides at (low) non-toxic doses of the individual chemicals is likely to be small, since the dose of pesticides to which humans are exposed is generally much lower than the NOAEL, at least through food.

x. *Some endpoints that have been studied in animals or in in vitro systems are relevant to groups in the population believed to be at higher risk than the general population. Such endpoints include developmental toxicity studies, endocrine and neurotoxic effects and genotoxicity studies. On the basis of limited information it seems likely that the default assumptions in relation to mixtures in children and pregnant and nursing mothers, would be the same as for the rest of the population."*

COT Assessment of the current research project

21. Important interactions between some toxicants are known to occur at relatively high doses (i.e. above their individual effect levels). Most of the evidence for such phenomena relates to pharmaceuticals. Exposures to chemical contaminants in food, consumer products and the environment are generally well below individual effect levels. Many of the mechanisms that underlie demonstrable interactions at high doses would not be expected to cause important interactions at the much lower doses used in this study. For example, major modification of metabolic activation or detoxification by enzyme induction or competitive inhibition is unlikely. The limited empirical evidence currently available provides no indication of important toxic interactions at low doses, although in some circumstances dose additivity can lead to effects from combinations of toxicants at doses of the individual compounds below their effect levels.

22. Testing for possible interactions between toxicants is complicated as often more than one measure of effect could be used, and additivity in relation to one effect measure will not necessarily imply additivity for another. For example, an effect on liver size could be characterised either by the average increase in liver weight at a given dose, or by the proportion of animals at that dose with more than a specified increase in liver weight. If individual doses of two compounds were just below those necessary to increase liver weight above the specified value, their combination could be additive in respect of average increase in liver weight (effect addition) but more than additive in respect of the proportion of animals with liver weights exceeding the specified increase (response addition). If another measure of effect were adopted, based on a linear measure of liver size (e.g. maximum "diameter" in mm), by definition this could not demonstrate additivity if effects on average liver weight (which varies as the cube of its linear dimensions) were additive.

23. In the new research project there was no evidence of interactions leading to overt toxicity with the "conventional" endpoints employed, and therefore the rationale for investigations at the genomic level was queried. Many of the endpoints studied have no clear role in toxicity and others may not be relevant to toxicity in humans.

For instance the measurement of induction of xenobiotic-metabolising enzymes was not focused on enzymes with a known role in toxicity, making it impossible to predict toxic effects that might result following induction of these enzymes. If the mode of action of induction for the chosen biomarkers was known to be a key event in the pathway leading to an adverse effect, there would be a far more plausible rationale for analysing the chosen gene. For example CYP2B induction could serve as a biomarker for activation of the constitutive androstane receptor (CAR), a key event in the hepatocarcinogenic effects of certain compounds. However, as analysis of the mode of action for the chosen biomarkers was not carried out, their role if any, in leading to adverse effects of the compounds (i.e. the four additives) is unknown.

24. The use of a very limited range of biomarkers, not necessarily reflecting adverse effects, meant that the dosing schedule used in this study might not have covered a sufficiently wide dose range to identify thresholds for adverse effects.

25. The study provided an opportunity to explore the use of transcriptomics in mixtures toxicology, but the design was not optimal and the rationale for the timing of these investigations at the genomic level was questioned. For transcriptomics analysis, samples are usually taken early in the study in order to identify direct effects of the treatment rather than secondary effects, for example resulting from pathological changes. In addition it is necessary to follow the time course of the changes from early on, and these should be anchored to some toxicological response to allow adequate interpretation ("phenotypic anchoring"). The timing of events will vary depending on the nature of the effects. At the present time, knowledge on the sequence of events in time is very limited, so that changes in the expression of certain genes could represent either a prelude to toxicity, homeostatic regulation or adaptation. Currently, it is often not possible to distinguish between these possibilities, and in the absence of any clear signs of toxicity, this becomes even more problematical. As time from the initial exposure increases, the possibility that any changes in gene expression may represent secondary effects, possibly due to homeostatic regulation, also increases. Hence, in-depth knowledge of the consequences of specific gene/pathway changes is required, not just qualitatively but also quantitatively. However, in the absence of any observable toxic response in the current study, secondary effects are unlikely.

26. For both propyl gallate and curcumin there were no changes in CYP levels on an individual basis, but small changes in gene expression were observed when these additives were tested in combination. In the absence of pathological changes, the significance of this is unclear.

27. The design allowed for testing of compatibility of the measured effects with predicted data for binary and quaternary mixtures, according to accepted principles in mixtures toxicology. A number of statistically significant deviations from combined effects predicted on the basis of effect additivity were observed. It was difficult from the way in which the data were reported to ascertain their biological significance. For example, information on the background variability in the responsive genes would have been of value, as would the extent to which allowance was made for the multiplicity of comparisons and whether the deviations observed were biologically coherent. However, as samples used for transcriptomics were pooled, such data

could not have been obtained. Dose-dependency of a number of the changes was limited. This made them difficult to interpret in the context of mixtures toxicology.

Interpretation

28. Members did not consider it surprising that in this study on food additives, deviations from effect additivity differed for liver weight and the various molecular markers. The small deviations for some of the metrics were considered to be of limited importance. In practical terms when exposures are at low doses, what matters is whether large deviations from effect additivity could occur that could lead to important toxicity well above any that would be expected from the individual components of a mixture. There was no indication of such a phenomenon in these data. The deviations from effect additivity for the various molecular markers were generally less than two-fold, but with a few (one *in vivo* for CP1A2 mRNA and two *in vitro* for BFC O-debenzylase activity) up to about eightfold.

29. In assessing the toxicity of mixtures, the effects of combined exposure can be determined either by direct study of the mixture or by prediction, based on assumptions about how the components of the mixture might interact. The latter strategy requires fewer resources and fewer animals. The present study demonstrated little deviation from effect additivity for "conventional" endpoints and did not reflect a potential for adverse health effects. In addition some of the hepatic changes may not be relevant to humans and this needs to be taken into account.

30. Because of the absence of overt toxicity at the doses given, findings relating to mode of combined action of the four additives investigated were of very limited value in exploring the possibility that these data could be used to predict additive or greater than additive, changes in toxicity.

31. The choice of compounds studied in this research was based on possible effects on the liver. It was a logical approach for an initial grouping to be based on morphological or adverse effects in the liver but the rationale for grouping these compounds on the basis of effects on xenobiotic-metabolising enzymes is less clear. Whereas some effects other than changes in xenobiotic-metabolising enzymes were measured *in vivo*, the hepatocyte studies only looked at cytotoxicity and changes in CYP mRNA levels and enzyme activities. Hence, the applicability of studies in hepatocytes to explore potentially adverse effects of mixtures cannot be determined from this study. Synergistic effects were found in mRNA expression levels with some mixtures that had no measurable effect on any other parameter. The biological relevance of such effects is not known and no adverse or other effect was observed in a 28-day toxicity study. However, if the mixture had been tested and such changes had been seen, there might be some basis for investigating further.

33. These results demonstrate that transcriptomics analyses cannot be used routinely in risk assessment of mixtures, since interpretation of the data, and the relevance to risk assessment are unclear unless they can be linked to conventional toxicological endpoints.

34. Results obtained for the compounds tested in this project did not suggest that these mixtures would lead to adverse effects in humans, or suggest important toxic

interactions at the low doses to which consumers would be exposed. However, due to limitations in the study, it was difficult to assess whether there might be a risk from low level combined exposures. This was because the choice of endpoints was often not informative of possible human health effects. No analysis of mode of action was undertaken and no toxicity data were generated *in vitro* with mixtures of the compounds. Comparisons of human intake with the maximum combined exposure when no adverse effects were detected might provide useful information on the risk of mixtures of these compounds. In addition, rather than focus on biochemical changes it might have been more productive to look for effects, such as morphological changes that might have been predicted to occur based on available information on the four compounds and their effects in the liver.

35. The results of this study do not suggest important toxic interactions at low doses for the additives tested.

Priorities for future research

36. Priorities for future research remain to test the basic assumptions of mixture toxicology, including the application of modelling approaches. It might be possible to use hepatocytes, though this approach requires further consideration to identify relevant endpoints. Compounds could be included on the basis of target organ (and/or cell type), mode of action (e.g. cytotoxicity, cell proliferation), or mechanism of action (e.g. direct-acting or activated by specific P450 enzymes; constitutive androstane receptor or the peroxisome proliferator-activated receptor α (PPARα) activator).

37. Endpoint selection should be based on relevant key events and on toxicological effects of relevance to human health. At least one mixture should comprise compounds all at or below their respective NOAELs. The applicability of transcriptomics (and other "omics" technologies) to this problem needs further investigation. Factors that should be taken into account include sample timing, dose-response analysis, statistical robustness, and background variability in expression and biological coherence, e.g. through pathway analysis.

38. Additionally it may be useful to test the robustness and try to reproduce the finding on CP1A2 that suggested a larger deviation from effect additivity.

Committee Conclusions

39. We consider that this study, which was substantial and complex, was carried out to high technical standards. However, at the doses studied, no overt toxicity was observed with the four additives either individually or in combination. Without using dose levels that demonstrate relevant toxicity or being able to extrapolate to such levels it is not possible to interpret the results of the transcriptomics studies with respect to implications for risk assessment.

40. We conclude that the new research does not raise concerns that combined exposure to the four compounds tested would pose a risk to health at doses individually below the Acceptable Daily Intakes.

41. Further work is needed to determine the applicability of transcriptomics in the risk assessment of mixtures.

COT statement 2008/09
December 2008

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