FINAL TECHNICAL REPORT

1. PROJECT DETAILS

FSA Project Code	:	T01040 and T01041 (formerly T01021)
Project Title	:	Development of methods for the assessment of the health effects from mixtures of food additives
Project Start Date	:	1 September 2001
Project End Date	:	31 August 2007
Report Date	:	17 December 2007, Revision on 2 November 2008 by Brian Lake, Followed by revision on 27 November by Rob Stierum.
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2. EXECUTIVE SUMMARY

The aim of this project was to develop methods to assess the health effects from mixtures of food additives. The project was formulated to address the FSA requirement for research into the combined effects of potentially harmful food chemicals as contained in the FSA Requirements Document 2001-2002, part 66B, page 19. From an evaluation of the available literature, the ILSI-Europe Acceptable Daily Intake Task Force identified a number of food additives where the possibility of joint actions or interactions occurring in selected target organs could not be excluded (Groten et al., 2000). With respect to the liver as a target organ a number of food additives were reported to produce liver enlargement, which was often associated with enzyme induction. Four such food additives, namely butylated hydroxytoluene (BHT; E321), curcumin (CC; E100), propyl gallate (PG; E310) and thiabendazole (TB; E233), were selected for this project. Studies were conducted in the rat using dietary administration with both the individual food additives and with mixtures of the food additives. In addition, *in vitro* studies with individual food additives and mixtures of the food additives were conducted in cultured rat and human hepatocytes. The purpose of the *in vitro* studies was to permit a comparison of the effects of the selected food additives in human and rat liver.

The overall aim of this project was to assess the joint actions and interactions between four food additives with the liver a target organ. The hypothesis tested in this project was thus whether treatment with BHT, CC, PG and PG would result in simply additive effects (i.e. non-interactions where the components of a mixture do not affect each others toxic response), or whether any interactions would occur, leading to either potentiation or antagonism of the effects of the individual compounds. The statistical design employed in theses studies permitted the assessment of both deviation from effect addition and deviation from dose addition. The terminology used in describing the possible combined actions of chemicals in a mixture has been considered by the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT). This terminology (COT, 1992; based on Cassee et al., 1999) is summarised below.

Concept of type of combined behaviour	Terms used by COT	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

We have used the term effect addition since calculations were based upon continuous variables

Gene expression profiling, RT-PCR and enzymatic assays were performed for selected CYPs and glutathione S-transferases. In combination with clinical endpoints in the in vivo studies, this molecular approach was chosen to allow for extrapolation of some of the *in vivo* findings in the 28 day rat studies towards rat hepatocytes in vitro, and subsequent interspecies extrapolation towards human hepatocytes. It is fully understood that not all of these molecular changes have to be necessarily related in a causal manner to the endpoints (liver hypertrophy). Also, this was not the purpose of these studies as described in the Scope of Work. However, this approach was chosen to provide answers towards the extrapolation of some of the mixture findings towards man, which would have been even more difficult to achieve if only clinical endpoints (e.g. body weight, liver weight) were obtained from the rat in vivo studies. Wherever possible, the mechanistic findings are discussed in light of possible physiological changes.

2.1 Rat in vivo Studies with Individual Food Additives

Studies were performed in the rat with the individual food additives to determine suitable dietary levels of the compounds for subsequent mixtures studies and to identify suitable biomarkers based upon genomics and molecular assays of effect of the compounds for use in the subsequent mixtures studies.

Each of the four food additives was administered in the diet to male Sprague-Dawley rats for 28 days at five dose levels ranging from 254 to 10154 ppm for BHT, from 254 to 10154 ppm for CC, from 203 to 6092 ppm for PG and from 102 to 5077 ppm for TB. At necropsy blood samples were taken for clinical chemistry analysis and the livers removed and samples taken for morphological examination, transcriptomics analysis, measurement of mRNA levels and biochemical analysis. The aim of the transcriptomics analysis within the single compound studies was to contribute to the identification of possible novel biomarkers for the subsequent mixtures studies. Thus, transcriptomics was performed to allow for the discovery of potential new molecular markers, in correlation with conventional findings, with which could be transferred from in vivo studies towards rat and human hepatocytes in vitro, to allow for interspecies extrapolations. Within the scope of this project it was however not the primary aims to fully obtain in depth understanding on the causality between these novel markers and the endpoints under study.

The major findings were as follows:

- None of the food additives appeared to be hepatotoxic at the dose levels examined.
- Both BHT and TB producing significant increases in relative liver weight.
- Treatment with BHT significantly increased hepatic microsomal total cytochrome P450 (CYP) content, 7-ethoxyresorufin O-deethylase and 7-pentoxyresorufin O-dependentylase activities, CYP2B1/2 apoprotein levels and CYP2B1 and CYP2B1/2 mRNA levels.
- TB increased the CYP-dependent enzyme activities measured and induced CYP1A2 and CYP2B1/2 apoprotein levels and CYP1A2, CYP2B1 and CYP2B1/2 mRNA levels.
- Both BHT and TB induced GSH *S*-transferase (GST) activities towards a number of substrates including 1-chloro-2,4,-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB).

- In contrast to BHT and TB, treatment with CC and PG produced only minor effects on the markers of hepatic xenobiotic metabolism examined.
- Transcriptomics analysis confirmed some of the changes in the phase I and Phase II xenobiotic metabolising enzyme described above
- Transcriptomics analysis further provided testable hypotheses that CC was a weak peroxisome proliferator and that TB was able to induce p53 protein. However, this was observed at the highest dose levels only. Further the dose levels employed in this study at which these effects were found were already higher than the currently established NOAELS for these two additives, indicating that these findings are of little toxicological concern. Therefore, these markers were not included in subsequent studies. Transcriptome findings further supported the inclusion of biomarkers related to phase I and phase II metabolism e.g CYP1A2, CYP2B1/2 mRNA and GST. The implications of these gene expression changes are discussed in light of some of the clinical findings pertaining to the single compound studies.

2.2. Rat in vivo Studies with Mixtures of Food Additives

The results of these studies with individual food additives were used to design the rat *in vivo* mixtures study. The design of the rat *in vivo* mixtures study comprised a control group and 27 experimental groups. For each of the four food additives the highest dose level was designated 100 units, being a fractional dose level of 100%, and was selected from the data obtained in the *in vivo* studies with the individual food additives. The selected highest (100 fractional dose level) dietary levels of BHT, CC, PG and TB were 3046, 4062, 3046 and 1750 ppm, respectively. Male rats were fed diets containing the individual food additives at dose levels of 25, 50 and 100 units, together with binary and quaternary mixtures of the food additives. The binary mixtures comprised all combinations with a total dosage of 50 (i.e. 25 + 25) and 100 (i.e. 50 + 50) units, whereas the quaternary mixtures comprised total dosages of 25 (i.e. 6.25 + 6.25 + 6.25 + 6.25), 50 (i.e. 12.5 + 12.5 + 12.5 + 12.5) and 100 (i.e. 25 + 25 + 25 + 25) units. Rats were fed the diets containing the food additives and mixtures of food additives for 28 days. At necropsy the livers were removed and samples taken for transcriptomics analysis, measurement of mRNA levels and biochemical analysis. Transcriptome analysis in the mixture study was performed to obtain insights if the observations made with conventional endpoints (e.g. body weight, liver weight), markers for phase I (CYP mRNA, protein levels and enzyme activity) and Phase II metabolizing enzymes (enzyme activities) were corroborated by gene expression

changes. Further, without the aim of redefining currently established NOAELs or claiming causalities, it was for the first time investigated in mixture toxicity studies of these additives, in how far the sensitivity of gene expression profiling relates to more conventional endpoints. This work was done to accommodate Objective 03. Gene expression analysis of samples from the rat in vivo mixtures study, as described in the Scope of Work entitled: Development of methods for the assessment of the health effects from mixtures of food additives-continuation 2006 (TNO part). The aim of the application of toxicogenomics in the mixture studies was slightly different from the application in the studies with single compounds, for which the main purpose was the discovery of single gene based markers for in vivo-in vitro and interpecies extrapolations.

The endpoints analysed for effects of the food additive mixtures comprised body weight, absolute and relative liver weight, total CYP content, 7-ethoxyresorufin O-deethylase and 7-pentoxyresorufin O-depentylase activities, CYP1A2 and CYP2B1 mRNA levels and GST activity towards CDNB and DCNB as substrates. The design allowed for testing of compatibility of the measured data with predicted data for binary and quaternary mixtures, according to classical principles in mixture toxicology: effect addition and dose addition. Effect addition or dissimilar joint action is non-interactive, namely, each chemical in the mixture does not affect another's toxicity. The modes of action and possibly the nature and site of toxic effect are likely to differ among the chemicals in the mixture. The toxicity of the mixture can be predicted from the dose-response curves obtained from the individual chemicals. Dose addition or similar joint action applies whenever chemicals produce similar but independent effects, so that one chemical can be substituted at a constant proportion of the other. In this case, the mode of action is believed to be similar. As a consequence, toxicity of a mixture can be predicted using a summation of the doses of each individual chemical after adjusting for the differences in potencies. Predicted data for mixtures were derived from additivity surface equations which were obtained from response curve modelling for the individual additives. If measured data for mixtures were significantly different from predicted data, under either the assumption of effect addition or dose addition, it was assumed that interactions were likely. Interactions were categorized in terms of antagonism being less than expected and synergism being more than expected. The major findings were as follows:

-With respect to liver weight and body weight, no interactions were present in binary and quaternary mixtures of the food additives BHT, CC, PG and TB. In most instances effect addition was applicable, meaning that the effects in mixtures could be directly predicted from

the summation of the magnitude of effects induced by individual additives. Current NOAELs for individual additives were not based upon data from the present study. Therefore comparison of the levels of additives resulting in the present mixture data with established safety levels for individual additives should be made with care, since interstudy differences may exist (different strains and dosage regimens). However it is worthwhile to mention that for body weight and liver weight, for which mostly effect addition applied, at dosages of individual additives in mixtures around the reported NOAEL or effect level from other studies, in this study, the magnitude of change compared to control did not exceed the observations made with individual additives.

-Clear interactions were observed in phase I metabolism, in particular at the level of CYP1A2 mRNA induction, as well as glutathione S-transferase enzyme activities. In some instances this was observed at dose levels at or around the NOAEL of individual additives, reported in other studies. However given the absence of interactions with respect to liver weight (and body weight) these findings likely imply that within mixtures these additives do influence each others metabolism, although except for CYP1A2, in general these effects are minor. Further, these interactions at the mechanistic level are certainly not sufficient to cause any interactions at the physiological levels, as revealed by liver weight or body weight, at least for a 28 day exposure duration in Sprague-Dawley rats.

2.3. Transcriptomics Analysis of the Hepatic Effects of Mixtures of Food Additives in the Rat

In addition to more conventional approaches to mixture toxicology described above, gene expression analysis was performed on liver samples obtained from the rat mixture study, by means of Affymetrix GeneChip Rat Expression 230A array analysis. The major findings were as follows:

-TB had the most profound effects on the hepatic gene expression profile, followed by BHT, PG and CC, as individual additives.

-Gene expression analysis in the mixture study described above indicated that in binary and quaternary mixtures TB also had the most dominant effects, in terms of induction of differential gene expression. Moreover, in mixtures containing BHT, addition of CC and PG did not further influence to any great extent the gene expression profile induced by BHT.

-Although CC and PG themselves had only modest effects on hepatic gene expression profiles, in combination in a mixture with each additive at 50% fractional dose level, profound gene expression changes were observed. However, no interactions were observed for CC PG mixture

at the level of liver weight and body weight. Thus these molecular changes do not cause physiological changes within this 28 day study.

- Analysis for enrichment of gene sets (T-profiler analysis) indicated that gene sets related to transcription were enriched –meaning genes within these gene sets were differentially expressed-, but not by the individual additives. This indicates the potential occurrence of a specific mixture effect at the molecular level of gene expression. Major interactions were not observed for this mixture, based upon conventional endpoints. Therefore, it remains to be determined if these mixture-specific gene expression changes could underlie development of effects at the physiological level in time, or if these gene expression changes are simply reversible. As outlined in the Scope of Work, detailed biological interpretation of the data was beyond the scope of these studies. Within the scope of these 28 day studies it is certainly evident that the mutual effects these additives had on each others gene expression profiles was not sufficient to cause any physiologically relevant interaction (e.g. on liver weight and body weight).

2.4. Rat and human in vitro Studies with Individual Food Additives

To allow for limited *in vitro* towards *in vivo* extrapolation and interspecies extrapolation of mixture effects if any, *in vitro* studies were performed with rat and human hepatocytes. Aim was to facilitate this extrapolation by inclusion of preselected markers related to phase I and phase II compound metabolism, since extrapolation of physiological endpoints (liver weight, body weight) to in vitro models is not possible. Genomics based markers (e.g. peroxisome proliferation, p53) were not included given the fact that, although certainly sensitive in relation to conventional endpoints, these markers were expected of being only activated at very high dose levels in man in relation to mechanisms of less toxicological relevance to man (peroxisome proliferation).

The *in vitro* studies comprised investigating the effects of the food additives and food additive mixtures in cultured rat and human hepatocytes. Initial studies were performed with the individual food additives in order to identify suitable culture medium concentrations and biomarkers of effect for the subsequent *in vitro* mixtures studies. Male Sprague-Dawley rat and human hepatocytes were treated for 72 hr with the individual food additives. The major findings were as follows:

-While BHT and TB were not overtly toxic to rat and human hepatocytes at culture medium concentrations of up to 200 µM, PG was cytotoxic at concentrations of 50 µM or greater and CC was cytotoxic at concentrations of >20 µM.
-In rat hepatocytes TB produced a marked induction of CYP1A2 mRNA levels, whereas BHT produced some induction of CYP2B1 mRNA levels. Both BHT and TB also induced 7-benzyloxy-4-trifluoromethylcoumarin (BFC) O-debenzylase activity in cultured rat hepatocytes.
-Studies with human hepatocytes demonstrated that TB produced a marked induction of CYP1A2 mRNA levels, with TB and BHT also inducing CYP2B6 and CYP3A4 mRNA levels.
-In contrast to BHT and TB, treatment with CC and PG did not result in any marked induction of CYP endpoints measured in both rat and human hepatocytes.

2.5. Rat and human in vitro Studies with Mixtures of Food Additives

The design of the rat hepatocyte mixtures study comprised control and 31 concentrations of either individual food additives or mixtures of food additives. The single food additive treatments comprised 5 and 100 µM BHT, 2, 7.5 and 15µM CC, 2 and 20 µM PG and 20 and 100 µM TB, whereas the six binary mixtures studied comprised the highest individual concentrations of the four food additives. Studies were also conducted with eight quaternary mixtures containing the lowest and highest concentrations of BHT, PG and TB together with 2 µM CC and eight quaternary mixtures containing the lowest and highest concentrations of BHT, PG and TB together with 7.5 µM CC. Rat hepatocytes were treated with the individual food additives and the food additive mixtures for 72 hr. The biomarkers of effect were CYP1A2 and CYP2B1 mRNA levels and BFC O-debenzylase activity.

The design of the human hepatocyte mixtures study comprised control and 27 concentrations of either individual food additives or mixtures of food additives. For each of the food additives the highest culture medium concentration was designated 100 units and was selected from the data obtained with the individual food additives. The selected highest (100 unit) medium concentrations of BHT, CC, PG and TB were 75, 7.5, 10 and 75 µM, respectively. Human hepatocytes were treated with the individual food additives at dose levels of 25, 50 and 100 units, together with binary and quaternary mixtures of the food additives. The binary mixtures comprised all combinations with a total dosage of 50 (i.e. 25 + 25) and 100 (i.e. 50 + 50) units, whereas the quaternary mixtures comprised total dosages of 25 (i.e. 6.25 + 6.25 + 6.25 + 6.25), 50 (i.e. 12.5 + 12.5 + 12.5 + 12.5) and 100 (i.e. 25 + 25 + 25 + 25) units. Human hepatocytes

were treated with the individual food additives and the food additive mixtures for 72 hr. The biomarkers of effect were CYP1A2, CYP2B6 and CYP3A4 mRNA levels.

The major findings were as follows:

-With respect to CYP1A2, antagonistic effects were observed in some of high dose level quaternary mixtures tested in rats *in vivo*, rat hepatocytes, as well as human hepatocytes. This interspecies and *in vitro* to *in vitro* extrapolation was not clearly possible for binary mixtures. Further, no such findings were observed in rats *in vivo* for 7-ethoxyresorufin O-deethylase activity, which is believed to be, next to CYP2C6 a marker for induction of CYP1A as well. Therefore, interactions are present at the level of transcription of CYP1A2, but these are possibly not further exerted at the level of enzyme activity.

-The comparison of the *in vitro* data for the expression of NaPB inducible CYPs such as CYP2B1 (rat in vitro, in vivo), CYP3A4 (human) and CYP2B6 (human), together with BFC O-debenzylase activity (rat), as marker for CYP2B (and CYP1A) activities, proved to be complicated in terms of *in vitro* to *in vivo* and interspecies extrapolations. Indeed interactions were observed in the *in vitro* models for some binary and quaternary mixtures for the expression of these enzymes, however, results were not compatible across species and the *in vitro-in vivo* boundary.

2.6. Conclusions

Altogether, this study indicates that in Sprague Dawley rats *in vivo* major interactions between BHT, CC, PG and TB, administered for 28 days in the diet, with respect to clinical endpoints as body weight and liver weight are unlikely, at least for the binary and quaternary combinations tested. In most instances, simple effect addition was applicable. In contrast, antagonism was observed for CYP1A2 mRNA expression and modest synergism was observed for phase II metabolism, represented as GST activities towards CDNB and DCNB. Gene expression analysis performed on liver samples obtained from the rat *in vivo* range finding studies with individual additives corroborated the mRNA expression findings for CYPs. Further, based upon the transcriptomics data, it was found a.o. that CC is a weak peroxisome proliferator and TB a weak inducer of p53 protein, however only at high dose levels which are likely to be irrelevant to man. Gene expression analysis also proved to be a useful method to identify the most dominant additive in these mixtures (TB) and to identify mixture-specific effects that were not noticed with biomarkers for CYP and GST mRNA expression and activity (for CC+PG). An attempt

was made extrapolate the rat *in vivo* mixture effects along the interspecies and *in vitro-in vivo* parallelogram approach, using rat and human hepatocytes cultured in vitro. This proved to be difficult, and was at best possible for quaternary mixtures employing CYP1A2 mRNA expression as biomarker. Given differences between the present study and earlier studies underlying the establishment for individual additives of NOAELs, and from these inferred ADIs as safety level in man, detailed interpretation of the present findings towards human risk assessment is not trivial. However, from a hazard perspective, the rat *in vivo* study seems to indicate that additional liver enlargement invoked by mixtures of these additives appears to be unlikely, even at high dose levels exceeding the established NOAEL. In contrast, interactions were observed at the level of Phase I and Phase II metabolism and transcriptomics indicated possible mixture effects. This indicates that these additives might influence each others metabolism or effects at the transcriptional level, however, it is clear that within the scope of these studies (28 day) these effects are not related to the occurrence of interactions at the more physiological level (liver and body weight).

3. INDEX AND GLOSSARY

3.1.INDEX *(Note: page numbers to be amended)*

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3.2. GLOSSARY

ADI	=	acceptable daily intake
ALT	=	alanine aminotransferase
AST	=	aspartate aminotransferase
BFC	=	7-benzyloxy-4-trifluoromethylcoumarin
BHT	=	butylated hydroxtoluene
BNF	=	β-naphthoflavone
CAR	=	constitutive androstane receptor
CC	=	curcumin
CDNB	=	1-chloro-2,4,-dinitrobenzene
CH	=	cumene hydroperoxide
CYP	=	cytochrome P450
DCNB	=	1,2-dichloro-4-nitrobenzene
DMSO	=	dimethyl sulphoxide
EA	=	ethacrynic acid
ENPP	=	1,2-epoxy-3-(*p*-nitrophenoxy)propane
g	=	gram
GSH	=	reduced glutathione
GST	=	GSH *S*-transferase
GSTM2	=	GST form mu 2
GSTP1	=	GST form pi 1
GSTT1	=	GST form theta 1
HPLC	=	high performance liquid chromatography
hr	=	hour
l	=	litre
LDH	=	lactate dehydrogenase
min	=	minute
mol	=	mole
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaPB	=	sodium phenobarbitone
NOAEL	=	No observed adverse effect level
palmitoyl-CoA	=	palmitoyl-coenzyme A
PCN	=	pregnenolone-16α-carbonitrile

PG	=	propyl gallate
PXR	=	pregnane X receptor
RIF	=	rifampicin
RT-PCR	=	reverse transcription-polymerase chain reaction
SD	=	standard deviation
SDH	=	sorbital dehydrogenase
SEM	=	standard error of the mean
TB	=	thiabendazole
TPBO	=	*trans*-4-phenyl-3-buten-2-one
UK HTB	=	UK Human Tissue Bank
UV	=	ultraviolet
v/v	=	volume/volume
w/v	=	weight/volume

4. AIMS AND OBJECTIVES OF THE INVESTIGATION

The overall aim of this project was to develop methods to assess the health effects from mixtures of food additives. The project was formulated to address the FSA requirement for research into the combined effects of potentially harmful food chemicals as contained in the FSA Requirements Document 2001-2002, part 66B, page 19. While the present project deals with the hepatic effects of food chemicals (see below), the general principles described and experimental procedures utilised may be applied to investigate the effects of mixtures of food chemicals on other target organs. One important feature of the present project is the use of both *in vivo* and *in vitro* systems. The *in vitro* studies were conducted with both rodent and human tissue in order to permit a direct comparison of the effects of the selected food chemicals in human and rat liver. Next to studying the effects of these additives as single compounds and within mixtures on liver weight and body weight, the purpose of these in vivo studies was to provide a set of markers related to phase I and phase II metabolism (GST), as well as additional markers derived from toxicogenomics experiments.

Food additives are permitted in the European Union on the basis that they present no hazard to the health of the consumer at the proposed level of use (Groten et al., 2000). Although intakes of individual additives arising from their permitted levels of use are considered to be safe, concerns have been expressed that the simultaneous intake of different food additives could be of potential significance for human health. An evaluation of the possibility of joint actions or interactions occurring between 350 food additives approved for use in the European Union was undertaken by the International Life Sciences Institute (ILSI)-Europe Acceptable Daily Intake Task Force (Groten et al., 2000). The three concepts of joint actions or interactions considered in this report were simple similar action (also known as simple joint action or dose addition), simple dissimilar action (also known as effect addition or response addition) and interaction where the combined effect may be either stronger (synergism, potentiation, supraadditivity) or weaker (antagonism, inhibition, subadditivity) than expected on the basis of either dose or response additivity (Bliss, 1939; Cassee et al., 1998; Groten et al., 2000; Loewe, 1953). The terminology used in describing the possible combined actions of chemicals in a mixture has been considered by the Committee on Toxicity of

Chemicals in Food, Consumer Products and the Environment (COT). This terminology (COT, 1992; based on Cassee et al., 1999) is summarised below.

Concept of type of combined behaviour	Terms used by COT	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

The ILSI-Europe Acceptable Daily Intake Task Force analysed the available data on 350 approved food additives to define the acceptable daily intake (ADI) and to determine the target organs for toxicity and the nature of any histopathological changes produced at doses above the no observed adverse effect level (NOAEL). From this initial screen some 65 food additives were identified which required more detailed analysis of the possibility of joint actions or interactions occurring in the common target organs. In most cases the possibility of joint actions or interactions occurring between theses food additives could be excluded on scientific grounds (Groten et al., 2000). However, for certain food additives the possibility of joint actions or interactions occurring in the liver, kidney and thyroid could not be excluded.

With respect to the liver the four food additives not excluded by the ILSI-Europe Acceptable Daily Intake Task Force comprised butylated hydroxytoluene (BHT; E321), curcumin (CC; E100), propyl gallate (PG; E310) and thiabendazole (TB; E233). The ADIs set by the European Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA) and the NOAELs reported by Groten et al. (2000) are shown below.

Additive	E Number	Uses	ADI (mg/kg body weight)	NOAEL[a]
Butylated hydroxytoluene (BHT)	E 321	Antioxidant	SCF: 0 -0.05 JECFA: 0 -0.3	25
Propyl gallate	E 310	Antioxidant	SCF: 0 – 0.5 JECFA: 0 -1.4	135
Curcumin	E 100	Colour	SCF: ADI not specified JECFA: 0 -3	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 JECFA: 0 – 0.1	10
Thiabendazole as a pesticide and veterinary medicine	N/A	Pesticide and veterinary medicine	EC: 0.1 JECFA: 0.1	10

[a]NOAEL values reported by Groten et al. (2000) in units of mg/kg/body weight/day.

Both BHT and PG are used as antioxidants, whereas CC is a major component of tumeric and TB is used as an anti-fungal agent for the treatment of fruits (Groten et al., 2000; Walton et al., 1999). TB is also used as a broad spectrum anthelmintic in various species and is also employed for the control of parasitic infection in humans (Walton et al., 1999). All these four food additives have been reported to produce liver enlargement, usually associated with enzyme induction (Groten et al., 2000; Walton et al., 1999). Some reported effects of BHT, CC, PG and TB on hepatic phase I (e.g. cytochrome P450 (CYP) forms) and II (e.g. GSH *S*-transferase (GST) and UDP glucuronosyltransferase (UGT) forms) xenobiotic metabolising enzymes are shown in Table 1. BHT is a well established inducer of CYP forms in the rat and mouse, whereas TB has been reported to induce CYP1A forms in cultured rabbit hepatocytes (Table 1). Both CC and PG have also been reported to affect hepatic xenobiotic metabolising enzyme activities.

Table 1. Effect of some food additives on hepatic xenobiotic metabolising enzyme activities

Food additive	Activity increased[a,b]
BHT (E321)	CYP1A1 and CYP1A2 CYP2A + CYP2B1 + CYP3A + CYP4A UGT GST
CC (E100)	Total CYP Cytochrome b_5 GST
PG (E310)	CYP1A2[c] Epoxide hydratase UGT GST
TB (E233)	CYP1A1 and CYP1A2

[a]Adapted from Groten et al. (2000). References cited by Groten et al. (2000) include: Aix et al. (1994); Depner et al. (1982); Manson et al. (1997); Rey-Grobellet et al. (1996); Sun and Fukuhara (1997); Sun et al. (1996) and Suzan and Rao (1992).
[b]Abbreviations are: CYP = cytochrome P450; GST = GSH *S*-transferase; UGT = UDPglucuronosyltransferase.
[c]Activity reduced.

The aim of the present project was to assess the joint actions and interactions between four food additives with the liver a target organ. The four food additives selected for this project were BHT, CC, PG and TB, as for these compounds the ILSI-Europe Acceptable Daily Intake Task Force concluded that combined actions or interactions might occur under experimental conditions (Groten et al., 2000). The hypothesis tested in this project was thus whether treatment with BHT, CC, PG and PG would result in simply additive effects (i.e. non-interactions where the components of a mixture do not affect each others toxic response), or whether any interactions would occur, leading to either potentiation or antagonism of the effects of the individual compounds. The statistical design employed in these studies permitted the assessment of both deviation from effect and addition and deviation from dose addition.

The project consisted of both *in vivo* and *in vitro* studies. In the *in vivo* studies the four food additives were administered in the diet to rats for 28 days and effects on a number of endpoints determined. The endpoints studied included clinical chemistry, liver

histology, transcriptomics, liver enzyme activities and mRNA levels. From the range finding studies with the individual food additives, suitable dietary levels were selected for *in vivo* mixtures studies employing binary and quaternary mixtures of the four food additives. The range finding studies were also used to identify suitable biomarkers of effect of the four food additives for use in the subsequent mixtures studies. Next to selected markers related to phase I and phase II metabolism, a toxicogenomics approach was pursued in the aim to propose additional markers. The rationale behind this was to generate a panel of markers which could be easily applied to both the in vivo mixture studies, and rat and human hepatocyte in vitro studies, both in the context of single compound and mixture studies. One of the reasons for this molecular approach, next to obtaining possible mechanistic insights, was that conventional markers (e.g. liver weight) can not directly extrapolated to hepatocyte-based in vitro systems. It should be stressed that these toxicogenomics studies were not designed to redefine the currently established NOAELs.

In the *in vitro* studies the effect of the four food additives on some markers of hepatic xenobiotic metabolism were studied in cultured rat and human hepatocytes. From initial range finding studies with the individual food additives, suitable medium concentrations were selected for *in vitro* mixtures studies employing binary and quaternary mixtures of the four food additives.

The results of the *in vivo* mixtures study with the four food additives in the rat and the *in vitro* studies in cultured rat and human hepatocytes are described in this report.

5. EXPERIMENTAL PROCEDURES

5.1. Materials

Butylated hydroxytoluene (BHT; purity 99.9%), propyl gallate (PG, purity 99.5%), thiabendazole (TB; purity 99.6%), sodium phenobarbitone (NaPB), β-naphthoflavone (BNF), rifampicin (RIF), pregnenolone-16α-carbonitrile (PCN), menadione (2-methyl-1,4-naphthoquinone), cadmium chloride, 7-ethoxyresorufin, 7-pentoxyresorufin, 7-benzyloxy-4-trifluoromethylcoumarin (BFC), testosterone, palmitoyl-CoA, 1-chloro-2,4,-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), cumene hydroperoxide (CH), ethacrynic acid (EA), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (ENPP), *trans*-4-phenyl-3-buten-2-one (TPBO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and sulphorhodamine B were obtained from Sigma-Aldrich Co. Ltd (Poole, Dorset, UK). Curcumin (CC; purity >98%) was obtained from Fisher Scientific UK (Loughborough, Leics, UK) and [4-^{14}C]Testosterone (specific activity 57.0 mCi/mmol) from GE Healthcare UK Ltd (Little Chalfont, Bucks, UK). Tissue culture media and materials were obtained from Invitrogen Ltd (Paisley, Scotland, UK), Type 1 collagen coated 96-well plates from Stratech Scientific Ltd. (Newmarket, Suffolk, UK) and Matrigel® Basement Membrane Matrix from Marathon Laboratory Supplies (London, UK). QIAGEN RNA*later*™, QIAGEN RNeasy® mini kits and QIAGEN Omniscript™ reverse transcriptase kits were obtained from QIAGEN Ltd (Crawley, West Sussex, UK) and TaqMan® Universal Master Mix Reagents, RNase inhibitor, random hexamer primers and an oligonucleotide primers and probe kit for human CYP3A4 from Applied Biosystems (Warrington, UK). The oligonucleotide primers and probes for rat CYP1A1, CYP1A2, CYP2B1, CYP2B1/2, CYP3A1, GSTM2, GSTP1, GSTT1 and albumin and human CYP1A2, CYP2B6 and albumin were synthesised by Applied Biosystems.

5.2. Animals and treatment

Male Sprague-Dawley rats were obtained from Harlan Olac (Bicester, Oxon) and were allowed free access to R and M No. 1 laboratory animal diet (Special Diets Services, Witham, Essex) and water. The animals were housed (3 or 5 rats per cage) in polypropylene cages with stainless steel grid tops and floors in rooms maintained at 22±3 °C with a relative humidity of 40-70% and were allowed to acclimatise to these conditions for at least 6 days before use. Male rats (around 6 weeks of age) were fed control diet or diets containing the individual food additives and mixtures of the food

additives for 28 days. Animal body weight and food consumption were monitored through all 28 days of the study and this data used to calculate mean daily intakes of the food additives in each of the experimental diets. At necropsy the animals were killed by exsanguination under carbon dioxide anaesthesia and blood samples collected for serum analysis. The livers were rapidly removed, weighed and sampled as follows:

a) Five 25-50 mg samples of the left lobe were removed, snap frozen in liquid nitrogen and stored at -80°C. These samples were used for the molecular analysis (TaqMan®) studies performed at BIBRA.

b) One approximately 700 mg sample from the left lobe was removed, snap frozen in liquid nitrogen and stored at -80°C. These samples were used for the transcriptomics studies performed at TNO.

c) Liver sections were taken and fixed in neutral buffered formalin. From the formalin fixed material paraffin sections of about 5 μm were cut and stained with haematoxylin and eosin. The morphological examination of the processed slides was performed by TNO.

d) The remainder of the liver was frozen at -80°C. These liver samples were used to prepare the subcellular fractions for assay of enzyme activities performed at BIBRA.

Serum enzyme activities, total protein, albumin, triglycerides and cholesterol were determined with a reaction rate analyser employing standard diagnostic test kits.

In addition to the above 28 days studies, some limited 3 day investigations were also performed where groups of rats were given control diet or diet containing the third and fifth highest dose levels of the four food additives (see Section 6.1.1.).

5.3. Analysis of BHT, CC, PG and TB in rodent diet

Samples of diet (10.00 g) were extracted by shaking and vortexing with 100 ml of HPLC grade methanol and the filtered supernatant diluted with methanol as required to give nominal concentrations of 0-200 μg/ml of each of the four food additives. Chromatography of 20 μl aliquots of the diluted extracts was performed with a 125 × 4 mm column of LiChroSpher 60 5μ RP-Select-B (Merck) protected by a 4 × 4 mm column of LiChroSPher 60 5μ RP-Select-B and mobile phases consisting of methanol (A), ultrapure water (B) and 1.0% (w/v) citric acid in ultrapure water (C).

Elution was achieved at a flow rate of 1.0 ml/min starting with 30% A, 65% B and 5% C for 10 min, changing to 95% A and 5% C over 10 min, holding at 95% A and 5% C for 4 min, changing to 30% A, 65% B and 5% C over 1 min and equilibrating at 30% A, 65% B and 5% C for 5 min before the next injection. The eluant was monitored at 280 nm. Retention times of TB, PG, CC and BHT were 5.56, 6.96, 10.85 and 13.38 min, respectively, with the limits of quantification in rodent diet being 0.05, 0.1, 1.25 and 12.5 ppm, respectively.

5.4. Biochemical analysis of rat liver

At necropsy, the remainder of the liver from each animal (i.e. after sampling for transcriptomics and mRNA studies) was stored at -80°C. Liver samples were thawed, homogenised and washed microsomal and cytosolic fractions prepared by differential centrifugation (Lake, 1987; Lake et al., 1998). Liver microsomal and cytosolic fraction protein content was determined by the method of Lowry et al. (1951) employing bovine serum albumin as standard. Whole liver homogenates were assayed for cyanide-insensitive palmitoyl-CoA oxidation activity and microsomal fractions were assayed for total CYP content and for activities of 7-ethoxyresorufin O-deethylase and 7-pentoxyresorufin O-dependentylase (Gray et al., 1983; Japenga et al., 1993; Lake et al., 1998). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western immunoblottting of microsomal fractions was performed as described previously (Japenga et al. 1993; Lake et al., 1998) employing a mini-PROTEAN II electrophoresis system. Immunoreactive bands were detected with enhanced chemiluminescence reagents. The specificities of the antipeptide antibodies to rat CYP1A1, CYP1A2 and CYP2B1/2 have been described previously (Edwards, 1998). Liver cytosols were assayed for GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide (CH), *trans*-4-phenyl-3-buten-2-one (TPBO), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (ENPP) as substrates. The assay methods employed were based on procedures described by Lawrence and Burk (1976) for GST activity towards cumene hydroperoxide and by Jakoby and coworkers for GST activity towards all the other substrates examined (Habig and Jakoby, 1981; Habig et al., 1974). Further, for selected samples obtained from TB treated animals p53 ELISA was performed. p53 nuclear oncoprotein levels in livers obtained from TB treated rats were determined using a p53 pan ELISA kit (Roche). The antibody employed, a peroxidase

labelled anti-h-p53 monoclonal antibody, detects a conserved, pantropic, denaturation stable antigenic determinant of the p53 protein for human, rat, and mouse. Sensitivity and detection range were $\geq$ 9 pg/ml and 9-1200 pg/ml, respectively. Frozen liver samples were diluted 1:2 (w/v) with RIPA buffer consisting of 20 mM Tris pH 7.5, 0.5 mM EDTA, 1.0% Nonidet P40, 0.5% sodium deoxycholate, 0.05% SDS, and the following protease inhibitors: 1 mM PMSF, 1 µg/ml Aprotinin, 2 µg/ml Leupeptin. Samples were homogenized on ice using a tissue homogenizer and centrifuged for 10 min at 10000 g, at 4 ^{o}C. ELISA performed on the resulting supernatants was done according to the Instruction Manual provided by the manufacturer. Protein determination was performed according to Bradford (1976) using Biorad reagents.

5.5. Measurement of CYP and GST mRNA levels in rat liver

Rat CYP and GST mRNA levels were determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) methodology (TaqMan®) employing a Perkin Elmer ABI Prism 7700 sequence detector (Applied Biosystems). At necropsy small samples of liver from the left lobe were snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from liver samples by the modified procedure of Chomczynski and coworkers (Chomczynski and Sacchi, 1987; Chomczynski and Mackey, 1995). Reverse transcription was performed with QIAGEN Omniscript™ Reverse transcriptase kits as described previously (Price et al., 2004). The TaqMan® primers and probe sets for rat CYP1A1, CYP1A2, CYP2B1, CYP2B1/2, GSTP1, GSTT1 and the procedure for real-time quantitative PCR were as described previously (Meredith et al., 2003; Price et al., 2004). The TaqMan® primers and probe set for GSTM2 were designed in house using Applied Biosystems Primer Express™ V 1.5 software. Nucleotide primers and probe sequences were checked against the NCBI BLAST database to ensure specificity for the selected gene. To normalise for RNA loading, levels of each of the CYP and GST mRNAs determined were expressed as a ratio to albumin mRNA which was co-amplified in a duplex reaction.

5.6. Transcriptomics studies on mRNA obtained from rat in vivo studies

5.6.1. Cy Dye base microarrays for rat in vivo studies with individual food additives

For samples obtained from the in vivo studies with individual additives, gene expression profiling was performed on cDNA based microarrays. Microarray experiments were

performed in line with the Minimum Information About a Microarray Experiment (MIAME) principles (Brazma et al., 2001), as agreed upon by the Microarray Gene Expression Society. Further details are available at http://www.ebi.ac.uk/arrayexpress/ under Identifier E-MEXP-116. In summary, selection of liver samples for transcriptome analysis was made after completion of the histopathological analysis and clinical chemistry. Liver samples obtained from treatment groups with dose levels at or around the currently established NOAELs and higher were included. Thus, for CC and PG, microarray analysis was performed on samples obtained from animals from the three highest dose groups (CC: 2034, 4165 and 10333 ppm; PG: 1464, 3280 and 6124 ppm). For BHT and TB, microarray analysis was performed on samples in those three highest dose groups where no changes in histology or clinical chemistry were observed (BHT: 744, 1457 and 2860 ppm; TB: 102, 240 and 758 ppm). Prior to isolation of total RNA, livers were homogenised with a mortar and pestle in liquid nitrogen. Frozen liver was weighed and 100 mg ($\pm$5%) of liver material obtained from each animal were pooled. For each dose group, equal weight amounts of liver were pooled from all animals within the same dose group (control group: 10 animals; compound groups: 6 animals). cDNA microarray analysis was performed using total RNA isolated from pooled liver material from animals within the same dose group. Gene expression studies using a pool of biological material have been performed before to study chemically-induced multiple gene expression changes. These studies show that transcriptome changes were comparable when cDNA microarray analysis was performed either on mRNA obtained from pooled liver from different animals versus mRNA obtained from one single animal (Bartosiewicz et al,. 2001a,b).The cDNA array approach applied here, using Cy5 and Cy3 fluorophore labelling, does not measure the absolute amount of mRNA derived from each gene, but instead generates a relative ratio measurement of the experimental sample as compared to a reference sample. To allow for comparison of expression patterns across different cDNA arrays, a reference RNA pool was introduced. This sample is referred to as Reference Control Sample (Heijne et al., 2003) and consisted of dissected organs of several rats (including liver (~50% w/w), kidneys, lungs, brains, thymus, testes, spleen, heart, and muscle tissues). All organs were homogenised together in liquid nitrogen identical as for the experimental samples. RNA isolation and quality check of RNA was performed as described elsewhere (Heijne et al., 2003). In order to obtain duplicate measurements for each sample, total RNA samples obtained from each pool of

liver samples were split into two. One half (25 μg) was labeled with Cy3 fluorophore and one half with Cy5 fluorophore and hybrized together with 25 μg of Reference Control Sample labelled with Cy5 or Cy3 respectively. Thus, for each RNA sample obtained from pooled liver material from each dose group, a Cy5 food additive/Cy3 reference and Cy3 food additive/Cy5 reference hybridisation was performed. The use of the dye-swap approach to obtain duplicate measurements has been used before in cDNA array analysis (Hegde et al., 2000; Heijne et al., 2003) and helps to correct potential gene-specific differences in Cy3 and Cy5 labeling, respectively. cDNA microarray slides were prepared as described in Heijne et al. (2003).The cDNA microarray used contained ~3000 different sequence verified rat cDNAs.(Research Genetics). Details on the generation of the cDNA microarray, RNA labelling reactions and hybridisation procedures were essentially as described by Heijne et al. (2003), except that scanning of the microarray slides was performed using a ScanArray Express Scanner (Packard Biosciences) and quantitation of Cy3 and Cy5 signals from each microarray spot was performed using Imagene™ analysis software from Biodiscovery, Inc.. Further details on experimental procedures employed are available in conjunction with the datasets at http://www.ebi.ac.uk/arrayexpress/ under Identifier E-MEXP-116.

5.6.2. Affymetrix GeneChip Rat Expression 230A array for rat in vivo studies with mixtures of food additives

With progress in the development of microarray analysis technology and decreasing pricing, it became feasible during the course of the project to perform Affymetrix GeneChip analysis on liver samples obtained from rat *in vivo* studies with mixtures of food additives. Advantage of this microarray platform is that the expression of a larger number of genes can be determined simultaneously. Further, the technical robustness of this platform is superior to cDNA Cy dye based arrays, e.g. no sensitivity of bleaching of Cy5 signal as a result of environmental ozone levels. RNA, meant for hybridization on Affymnetrix GeneChip arrays was processed according to the GeneChip One-Cycle Eukaryotic Target Labeling Assay (GeneChip® Expression Analysis Technical Manual, 2004, http://www.affymetrix.com/support/technical/ manual/expression_manual.affx). cRNAs were hybridized to Affymetrix GeneChip Rat 230A arrays , which features a total number of annotated 15923 probe sets. Finally, probe set intensities were detected with the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA).

5.7. Bioinformatics

5.7.1. Bioinformatics analysis performed on microarray data obtained from rat in vivo studies with individual food additives

As with the differences in array platforms employed for rat *in vivo* studies with individual additives and mixtures, with progress in bioinformatics during the past 5 years, differential bioinformatics approaches were employed to the array datasets from these studies as well. For cDNA microarray data obtained from *in vivo* experiments with individual food additives, experimental conditions were recorded into TNKnowBase, a in-house developed microarray data analysis application (Conesa et al,. 2001), which was available at TNO at the time of experimentation. The same data analysis platform was used to store and analyze gene expression data. TNKnowBase uses the SAS® statistical package accessed through SAS Enterprise Guide® for data processing and visualization. For each correctly measured spot on the microarray, the local background intensity was subtracted from the signal intensity. Fluorescence intensity in control spots that account for non-specific hybridization and background fluorescence were used to determine a minimal signal intensity threshold value of 1.5 for the two channels. For each hybridisation food additive/reference ratios were calculated, ^{2}log transformed, normalised using the Lowess normalization algorithm and scaled among slides (Yang et al., 2002). This procedure yielded the final gene expression data set, containing for each gene an expression value, as observed in the experimental sample, relative to the common reference control sample. Main purpose of the application of toxicogenomics to the rat *in vivo* study with individual additives was to find single gene biomarkers as candidates for further biochemical characterisation in order to show their possible involvement into the mode of action of these food additives, as opposed to finding for example clusters of co-regulated genes or commonalities between induced overall transcriptome changes. Therefore, a highly stringent selection procedure was applied to the final gene expression data set, to search for dose dependent changes in gene expression, upon administration of each additive. Gene expression changes were considered relevant whenever the following criteria were met:

1. A maximum of one missing value per dose response curve, meaning for each dye combination 3 out of 4 ratios present

2. A maximum coefficient of variation for ratios in the dye swap replicates of 0.5, meaning the dose response trends observed for both dye swap experiments should be similar
3. At least a 1.5 fold difference in gene expression between any of the doses per compound, to select for genes with a certain (1.5-fold) minimum magnitude of change in expression across the dose range tested.
4. Next to this selection procedure, particular attention was paid to the responses of Phase I and Phase II xenobiotic metabolising enzymes, since previous findings indicated effects of these additives on drug metabolising enzymes (Manson et al., 1997a; Walton et al., 1999). To make supplementary data available to the research community, all details on microarray design, experimental design, samples and RNA extracts used, hybridisation procedures and parameters used and measurement data were uploaded to the ArrayExpress database using the MIAMExpress online submission tool at the European Bioinformatics Institute. The SAS Enterprise Guide® tool was used to create the Array Description File (ADF), the Raw Data Files for each microarray combining the Cy3 and Cy5 data output generated by the Imagene™ Scanner, and the Final Gene Expression Data File containing all gene identifiers and their responses with each treatment. This additional information and supplementary data are available at http://www.ebi.ac.uk/arrayexpress/ under Identifier E-MEXP-116.

5.7.2. Bioinformatics analysis performed on microarray data obtained from rat in vivo studies with mixtures of food additives

RNA samples isolated from the livers obtained from the rat in vivo studies with mixtures of food additives were analyzed on 51 Affymetrix GeneChip Rat Expression 230A (RAE230A) arrays. This chip features a total number of annotated 15923 probe sets. Gene expression measurements obtained from 51 Affymetrix GeneChip Rat Expression 230A (RAE230A) microarrays were processed according to the data analysis pipeline shown below in Fig. 2. The quality control (QC) of the 51 microarrays was performed by assessment of various parameters that indicate the overall quality of microarray experiments. This included the evaluation of the Affymetrix MAS5 based criteria and a number of additional quality parameters. In addition, the outlier identification, to detect any aberrant micorarrays, in relation to the majority of the microarrays within the experiment was performed by correlation analyses of replicate experiments. In summary, the following parameters were evaluated in the QC analysis: (I) MAS5 quality control

Figure 2. Array analysis workflow

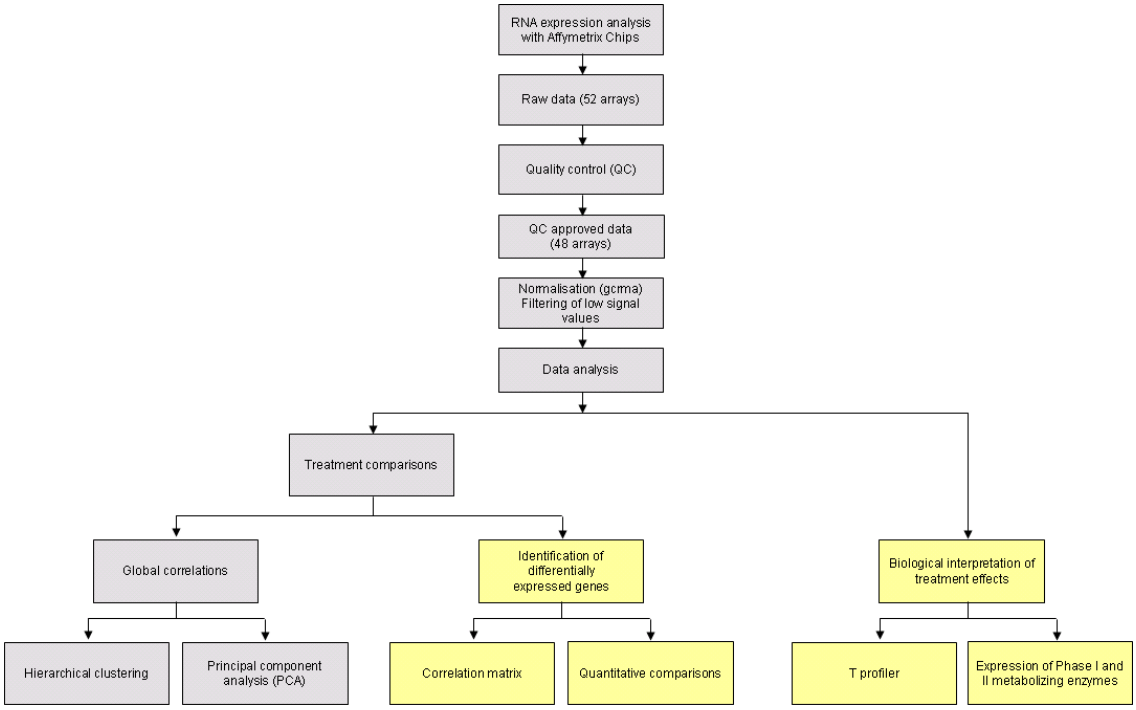

Fig. 2. Bioinformatics analysis workflow of gene expression analysis performed on liver samples obtained from rats treated for 28 days with BHT, CC, PG and TB.

criteria (quality plot with distribution of scaling factors; percentage of present calls; intensity of the background signals; 3' to 5' ratio of signal intensity for internal controls transcripts (β-actin, GAPDH); signal intensity of external controls transcripts (BioB, BioC, CreX); (II) Additional quality control criteria (visual inspection of image quality, including images of .CEL-files and the weight and residual FitPLM-plots; distribution of differences in the relative log expression values (RLE plots); distribution of the normalised unscaled standard errors (NUSE plots); distribution of signal intensities per array before and after normalisation (density distribution plots); distribution and variation in signal intensities before and after normalisation (box-plots); RNA digestion plots; MVA plots (before and after normalisation); intensity correlations (before and after normalisation)); (III) Outlier identification methods (after normalisation), to identify microarrays that are considered to be outliers (correlation analyses between replicate experiments; principal component analysis; hierarchical clustering). Quality control justified that selection of microarrays for further processing was primarily based on replicate correlation analysis and it resulted in the selection of 48 out of 51 microarray experiments that were considered to be of good quality for further bioinformatics

analyses. After obtaining the experimental set that passed the quality control (48 of 51 arrays), data were normalised using the gcRMA normalisation algorithm. Subsequently, data was subjected to a filtering procedure that is based on the flag values, describing for each of the individual genes their presence (present, absent, marginal). Only those transcripts that are called present in at least 1 of 48 arrays are taken for further analyses. This resulted in the reduction of the number of the probe sets from 15923 to 10906. After quality control and normalisation/filtering, data analysis was performed to understand the effects of exposure to mixtures of additives on global hepatic gene expression profiles (Figure 2, left part). To gain insight into relations between effects of different treatments on gene expression, comparisons were performed on the global gene expression level, being the expression of all genes present on the microarray, and on the level of differentially expressed genes between control and each of the treatment samples. For the global comparisons of treatments, expression values of replicate experiments were averaged and the total gene expression profile (i.e. 10968 probe sets that pass filter) for each treatment were subjected to hierarchical clustering (Pearson correlation, average linkage) and principal components analysis (mean centering and scaling). Identification of differentially expressed genes between control set and each of the treatments was performed using Cyber-T software XXX (Baldi and Long, 2001)(Baldi and Long 2001). Cyber-T algorithm employs statistical analyses based on regularized t-tests that use a Bayesian estimate of the variance among gene measurements within an experiment. To increase the robustness of differential expression analysis, additional fold change threshold was applied. Probesets with raw p-values smaller than 0.01 and with average absolute fold change greater than 1.5 are called significant. To further investigate if mixture effects are likely to occur and if mixture effects are primarily due to one compound, lists of differentially expressed genes obtained from Cyber-T analysis were compared with one another. This is indicated in Figure 2 in the rectangle identified as differentially expressed genes and onwards below. Venn Diagrams were created to look for overlap and dissimilarity in gene expression, with emphasis on genes exclusively expressed in the mixtures only, the sum of genes expressed in each of the individual compounds only and genes expressed in both the mixture and any of the individual compounds.

To further understand the correlation/distinction in gene expression profiles between the different treatments, genes that were differentially expressed in any of the treatments based on application of the Cyber-T algorithm (3538 genes in total) were used to calculate correlation coefficients in all pair wise combinations. This is indicated in Fig. 2 in the rectangle identified as 'correlation matrix'. In addition to the Original Scope of Work submitted to FSA in April 2006, a limited biological interpretation of treatment effects was performed. This involves limited activities towards biological analysis of gene expression data in single compounds and mixtures. To discover biological processes that are affected by treatment with BHT, CC, PG, TB and their mixtures, functional analysis of gene expression changes was performed using T-profiler (Boorsma et al., 2005). This tool enables calculation of significance (t-value) of the relative upregulation (positive t-values) or downregulation (negative t-values) of genes, compared to control, within a predefined group that either refers to a biological processes (P), molecular function (F) or cellular component (C); groups of genes classified by the gene ontology (GO) consortium (www.geneontology.org), in comparison to the total gene expression set. This analysis provides information which biological processes are changed upon exposure to additives and mixtures thereof.

5.8. Rat hepatocytes

Hepatocytes were obtained from male Sprague-Dawley rats (body weight 233 ± 12g, mean ± SEM, n=10) by a collagenase perfusion technique described previously (Gray et al., 1983). Viability (90 ± 1%, mean ± SEM, n=10) was determined by trypan blue exclusion. Isolated hepatocytes were seeded in Type 1 collagen coated 96-well plates at a density of 30,000 viable cells per well per 200 μl of culture medium. The culture medium consisted of Williams' Medium E containing 5% fetal calf serum, 1 μM bovine insulin, 0.1 μM dexamethasone and other additions (Price et al., 2008). Hepatocytes were cultured at 37°C in a humidified incubator under an atmosphere of 95% air/5% CO_2. After a 1 hr attachment period, the medium was removed and replaced with 100 μl/well of ice-cold serum free medium containing 0.2 mg/ml Matrigel® Basement Membrane Matrix. The cells were returned to the incubator and after 1 hr an additional 100 μl of serum free Williams' Medium E, containing all the above additions, added to each well. After approximately 24 hr the medium was removed and replaced with fresh medium.

Treatment was commenced after approximately 48 hr by replacing the medium with medium containing the test compounds.

5.9. Human hepatocytes

Human hepatocytes were obtained from UK Human Tissue Bank (HTB) (De Montfort University, Leicester, UK). The cells were isolated from pieces of human liver obtained with informed consent from subjects undergoing liver resections. Human hepatocytes were seeded in collagen coated 96-well plates at a density of 30,000 viable cells per well per 200 μl of standard UK HTB culture medium containing insulin and dexamethasone. The cells were cultured overnight at 37°C in a humidified incubator under an atmosphere of 95% air/5% CO_2 in the UK HTB laboratories. The 96-well plates of human hepatocytes were transported by courier the following morning to either BIBRA or LFI. On arrival the culture medium was removed and replaced with 100 μl/well of ice-cold serum free medium containing 0.2 mg/ml Matrigel®. The culture medium consisted of Ham F-12/ Williams' Medium E (1:1, v/v) containing 1 μM human recombinant insulin, 0.1 μM dexamethasone and other additions (Price et al., 2008). The cells were cultured at 37°C in a humidified incubator under an atmosphere of 95% air/5% CO_2. After 1 hr an additional 100 μl of the above culture medium was added to each well. Subsequently the medium was removed and replaced with fresh medium at 24 hr intervals. Treatment was commenced after approximately 24 or 48 hr by replacing the medium with medium containing the test compounds.

5.10. Treatment of hepatocytes

Rat and human hepatocytes were treated with the food additives and reference compounds (CYP inducers and cytotoxins) for 72 hr, the medium being changed after approximately 24 and 48 hr of culture. At each medium change fresh solutions of BHT, CC, PG, TB and the reference compounds were prepared in tissue culture medium. Stock solutions of BHT, CC, PG, TB, BNF, RIF, PCN and menadione of 1000 times the required final concentration in tissue culture medium were prepared in DMSO and aliquots stored at -20ºC. A stock solution of 20 mM cadmium chloride was prepared in filter sterilised deionised water and aliquots stored at -20ºC. Frozen aliquots of BHT, CC, PG, TB and the reference compounds were thawed only once to prepare dosing solutions by mixing with tissue culture medium. Fresh solutions of NaPB in tissue culture medium

were prepared for each day of dosing. DMSO was added at a concentration of 1 µl/ml to the control medium and the media containing NaPB and cadmium chloride. The final DMSO concentration was thus 0.1% (v/v) in all culture media including the control medium.

For the rat hepatocyte food additive mixtures studies (see Section 6.7.) stock solutions of 200 mM BHT, 30 mM CC, 40 mM PG and 200mM TB were prepared in DMSO. Aliquots of these solutions were diluted with DMSO to obtain solutions of 5 mM BHT, 2 mM CC, 2 mM PG and 20 mM TB. As some cytotoxicity was observed (see Section 6.7.), a 15 mM CC solution in DMSO was also prepared from the 30 mM CC stock DMSO solution. The various food additive solutions were mixed together and with DMSO as appropriate in order to prepare all the required single food additive DMSO solutions and the binary and quaternary food additive mixtures solutions in DMSO (see Section 6.7.). Aliquots of all these solutions were stored at -20ºC and were thawed only once to prepare dosing solutions by mixing with tissue culture medium. Due to food additive solubility considerations, the final DMSO concentration in the culture medium for the rat hepatocyte mixtures studies was 0.2% (v/v) in all culture media including the control medium (i.e. 2 µl DMSO solution was added to 1 ml of culture medium).

For the human hepatocyte food additive mixtures studies (see Section 6.10.) stock solutions of 300 mM BHT, 30 mM CC, 40 mM PG and 300mM TB were prepared in DMSO. Aliquots of these solutions were diluted with DMSO to obtain solutions of 75 and 150 mM BHT, 7.5 and 15 mM CC, 10 and 20 mM PG and 75 and 150 mM TB. The various food additive solutions were mixed together and with DMSO as appropriate in order to prepare all the required single food additive DMSO solutions and the binary and quaternary food additive mixtures solutions in DMSO (see Section 6.10.). Aliquots of all these solutions were stored at -20ºC and were thawed only once to prepare dosing solutions by mixing with tissue culture medium. The final DMSO concentration in the culture medium for the human hepatocyte mixtures studies was 0.1% (v/v) in all culture media including the control medium.

5.11. Assay of cytotoxicity and CYP enzyme activities in cultured hepatocytes

At the end of the treatment period the medium was removed from the plates and the cells washed at 37ºC with 200 µl/well of RPMI 1640 medium (phenol red free). To assay

cytotoxicity, the wash medium was removed and replaced with 40 µl/well of RPMI 1640 medium containing 2 mg/ml MTT. After a 30 min incubation at 37ºC the reaction was terminated by the addition of 100 µl/well of 0.1 M HCl in isopropanol containing 10% (w/v) Triton X-100. The plates were mixed on a gyratory shaker to extract the MTT formazan product and the absorbance of each well determined at 570 nm. To assay 7-ethoxyresorufin O-deethylase activity, the wash medium was removed and replaced with 100 µl/well of RPMI 1640 medium (phenol red free) containing 8 µM 7-ethoxyresorufin and 10 µM dicumarol. Stock solutions of 7-ethoxyresorufin and dicumarol were prepared in DMSO and added to the medium at 37ºC so that the final DMSO concentration was 0.45% (v/v). After a 40 min incubation at 37ºC, a 75 µl aliquot of the medium was removed from each well into a V-bottomed 96-well plate and the plate stored at -80ºC prior to analysis. The plates were thawed and levels of the resorufin product determined after enzymatic hydrolysis as described by Price et al. (2000). BFC O-debenzylase activity in rat hepatocytes (incubation time 60 min) was determined by the same procedure as for 7-ethoxyresorufin O-deethylase, except the substrate solution contained 50 µM BFC (added in DMSO, final concentration 0.4% v/v). Levels of the 7-hydroxy-4-trifluoromethylcoumarin product were determined after enzymatic hydrolysis (Price et al., 2000). To determine testosterone 6β-hydroxylase activity, the wash medium was removed and replaced with 100 µl/well of RPMI 1640 medium (phenol red free) containing 250 µM [4-^{14}C]testosterone (0.4 µCi/well, added in DMSO, final concentration 0.45%). At the end of the incubation period (rat hepatocytes 20 min, human hepatocytes 25-40 min) the medium was removed from each well and stored at - 80º C prior to analysis of the [4-^{14}C]6β-hydroxytestosterone product by HPLC (Lake et al., 1998). At the end of the incubations with the CYP substrates, the medium was removed and the cells fixed by adding 100 µl/well of 10% (w/v) trichloroacetic acid prior to determination of hepatocyte protein content (Price et al., 2000).

5.12. Measurement of CYP mRNA levels in cultured hepatocytes

Rat and human CYP mRNA levels were determined by real-time quantitative RT-PCR methodology (TaqMan®). At the end of the treatment period the medium was removed and 100 µl of Qiagen RNA*later*™ reagent added to each well. Total RNA was extracted from cultured rat and human hepatocytes using Qiagen RNeasy™ mini kits and reverse transcription performed as described previously (Meredith et al., 2003). To provide

sufficient total RNA for analysis four replicate wells for each treatment (i.e. control and each concentration of each test compound studied) were pooled. The primers and probe sets for rat CYP1A2, CYP2B1, CYP3A1 and albumin were as described previously (Meredith et al., 2003), whereas the primers and probe set for human CYP3A4 was obtained from Applied Biosystems. Primers and probes for human CYP1A2, CYP2B6 and albumin were designed using Primer Express™ v.1.5 software (Applied Biosystems) and are reported elsewhere (Price et al., 2008). The RNA sequences of CYP1A2 (Accession No. 6470142), CYP2B6 (Accession No. 6470136) and albumin (Accession No. 28591) were obtained from GenBank. Nucleotide primers and probe sequences were checked against the NCBI BLAST database for specificity to the selected gene. Real-time quantitative PCR for rat CYP and albumin mRNA levels and human CYP and albumin mRNA levels was performed employing a TaqMan ABI PRISM 7700 sequence detector system (Applied Biosystems) as determined previously (Meredith et al., 2003). To normalise for RNA loading, levels of each of the rat and human CYP mRNAs determined were expressed as a ratio to albumin mRNA which was co-amplified in a duplex reaction.

5.13. Statistical analysis

5.13.1. Statistical analysis of data from in vivo and in vitro studies with individual food additives

Statistical analysis of data from the rat *in vivo* studies and the rat and human hepatocyte *in vitro* studies with the individual food additives was performed by one one-way analysis of variance. Comparisons between means were made using the least significant difference test.

5.13.2 Statistical analysis of data from rat in vivo study with mixtures of food additives to infer mixtures effects

In order to statistically test according to basic principles in mixture toxicology, that is test for deviations from effect addition or dose addition and by doing so propose interactions, the study was performed according to a four compound mixture design represented in Table 15b. At the central basis of these statistical tests is the comparison between observed data for mixture groups and predicted data from an effect additivity surface, under the assumption that effect addition or dose addition is applicable. The design was

such that for binary mixtures and quaternary mixtures, statistical inference of the observed mixture data with predicted data based on individual compounds could indicate dose addition, effect addition or interaction, the latter being a stronger (synergism) or weaker (antagonism) effect than could be expected based upon effect addition or dose addition. The fractional dosage ‘f’ in Table 15b is defined as the dose level expressed as percentage of the maximum nominal dose level employed for each individual additive. The nominal dose level 'n' indicates nominal or target dosage: the dose level intended to be administered; the highest nominal dose of each compound corresponds to a fractional dose level of 100. The actual dose level employed in the study 'a' indicates actual dosage, the dose level experimentally determined by means of HPLC in the diet. The actual dose levels, or more precisely, the actual fractional dose levels calculated from these in relation to the maximum actual dose level employed, are used in the statistical analyses.

The representation of the design in Table 15b shows a division of the experimental groups into four sets:

- Set 1 (groups 1-13): control plus single compound exposures.
- Set 2 (groups 14-20): binary and quaternary mixtures with a total dosage of 100, obtained by adding up the fractional dosages of the respective compounds.
- Set 3 (groups 21-27): binary and quaternary mixtures with a total dosage of 50, obtained by adding up the fractional dosages of the respective compounds.
- In addition, a quaternary mixture with a total dosage of 25 obtained by adding up the fractional dosages of the respective compounds is included (group 28).

Data obtained from set 2 and 3, together with the single dosage groups at top dose (groups 4, 7, 10 and 13), and at middle dose (groups 3, 6, 9, and 12), respectively can be used for testing of deviations from effect addition and dose addition. In Figure 3 below, a visualisation is provided to further clarify the design, in this case for binary mixtures. This is a representation of all points involving just two of the compounds. In this representation, the design point in the upper left corner represents the control group. Design points on the bold axes are single dosages. There are two binary mixtures for each pair of compounds. As there are six pairs of compounds, a total of 12 binary mixtures are included in the study design. Such a design allows for comparison of observed responses in binary mixtures with predicted responses, based on an effect-additivity surface predicted from the individual compound exposures, thus allowing for

the statistical inference for deviation from *effect* addition (solid lines). The points connected with dashed lines allows for the comparison of single dose groups at a defined fractional dose level, with binary mixtures containing for each of these compounds half of the defined fractional dose level in order to statistically infer deviations from dose addition.

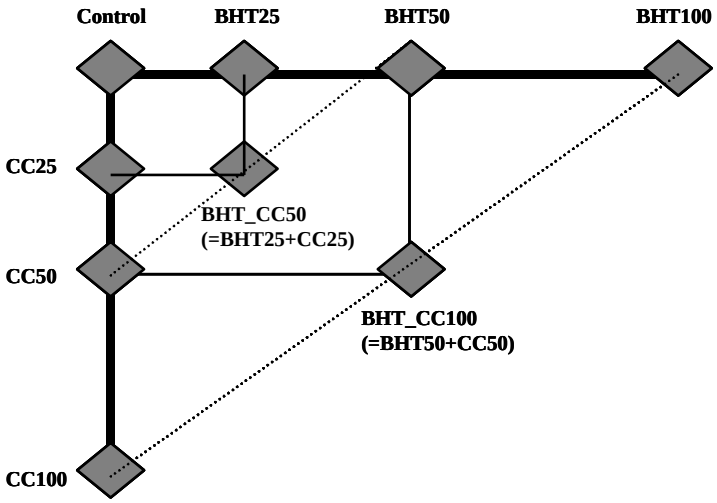

Figure 3. Graph illustrating the principle of the experimental design employed for the rat *in vivo* mixture study

Fig. 3. Representation of part of the experimental design, as example binary mixtures of BHT and CC. Solid lines: comparison of binary mixtures with single compounds refers to testing for deviation of effect addition/dissimilar joint action. Dashed lines: comparison of binary mixtures with single compounds refers to testing for deviation of dose addition/similar joint action. If both statistical inferences indicate that neither effect addition, nor dose addition applies, interactions are likely to occur, at least within the dose ranges selected.

For each of the parameters the following statistical procedures were followed: calculation of fractional dosages from actual dose levels, check on outliers in the data. Calculation of dose response curves for individual additives, assessment of the effect additivity surface equations using the curves for individual additives, testing for deviation from effect addition by comparison of observed and expected effects, based on the additivity surface, followed by more detailed assessment of the non-effect addition (e.g. in terms of linearity of the possible interaction), and finally deviation from dose addition are described in the supplementary data. Comparison of measured values with expected values, based on the additivity surfaces, under the assumption of effect addition or dose addition were

performed by means of Student t-test. Further extensive details on the statistical approach with guidance to data, are described in supplementary data in the document 'Supplementary outline on statistical analysis, tables, results and guidance to supplementary supplementary electronic files' (file: Supplementary statistics.doc) , which is included as the Annex Supplementary statistics at the end of this Report. Statistical calculations were done in GenStat Release 7.1 (PC/Windows 2000) Copyright 2003, Lawes Agricultural Trust (Rothamsted Experimental Station). Desired maximum target intakes (nominal dosages) for each additive and from these, derived fractional dosages, were based on initial single-compound range finding studies, in which dose range observations were generated for the same parameters as determined in the present study (Price et al,. 2004; Stierum *et al.,* 2008). The actual dietary levels of food additives in the diet were determined by means of HPLC analysis essentially as described in (Price *et al.* 2004) to check for consistency with expected nominal dietary levels.

In summary, the 28-group design outlined in Table 15b permits for each parameter the following issues to be addressed by statistical tests:

1) Calculation of an effect additivity surface for mixture combinations based on the individual additive dose groups (groups 1-13).
2) Checking of groups 14-27 on compatibility with effect additivity surface.
3) Prediction of quaternary mixtures from effect additivity surface and interactions calculated from binary compounds.
4) Prediction of the response in binary mixtures containing additives at 25% fractional dose level each, from the additivity surface and interactions calculated from the binary compounds at 25% fractional dose level.
5) Prediction of the response in binary mixtures containing additives at 50% fractional dose level each, from the additivity surface and interactions calculated from the binary compounds at 50% fractional dose level.
6) Prediction of binary mixtures from single compounds with the same total concentration (see dashed lines in the figure).
7) Prediction of quaternary mixtures from single compounds and binary mixtures with the same total concentration.

Issues 1-5 allow for the assessment of deviation from effect addition; issues 6 and 7 allow for the assessment of deviation from dose addition.

5.13.3. Statistical analysis of data from rat hepatocyte studies with mixtures of food additives to infer mixtures effects

Statistical testing for mixture effects with rat hepatocytes was confined to testing for deviation of effect addition only. This was done essentially similar as described above under paragraph for 5.13.2. Statistical analysis of data from rat *in vivo* study with mixtures of food additives to infer mixtures effects. The following steps were involved:

- outlier identification in the data
- calculation and check of the dose response curves for individual additives
- Establishment of Additivity Surface Equations for mixtures to estimate the predicted values
- Student t-test to test for significant differences between the measured and the predicted values, the latter based upon the Additivity Surface Equations

Details on the final design chosen were contingent upon initial pilot experiments (e.g. cytotoxicity) and are therefore further described in the results section in paragraph 6.7.1. Design of rat in vitro mixtures study and outlined in Table 38. Parameters involved in the rat *in vitro* mixtures experiments and statistical analysis for mixture effects included CYP1A2 and CYP2B1 mRNA expression levels as well as 7-benzyloxy-4-trifluormethylcoumarin (BFC) O-debenzylase activity.

5.13.4. Statistical analysis of data from human hepatocyte studies with mixtures of food additives to infer mixtures effects

Statistical testing for mixture effects with human hepatocytes was confined to testing for deviation of effect addition. This was done essentially similar as described above under paragraph for 5.13.3. Statistical analysis of data from rat hepatocyte studies with mixtures of food additives to infer mixtures effects. Details on the final design chosen were contingent upon initial pilot experiments and are therefore further described in the results section in paragraph 6.10.1. Design of human in vitro mixtures study and outlined in Table 50. Parameters involved in the rat *in vitro* mixtures experiments and statistical analysis for mixture effects included CYP1A2, CYP2B6 and CYP3A4 mRNA expression levels.

6. RESULTS

6.1. Rat in vivo Studies with Individual Food Additives

6.1.1. Design of study with individual food additives

The rationale for the selection of the four food additives, namely butylated hydroxytoluene (BHT), curcumin (CC), propyl gallate (PG) and thiabendazole (TB), has been described in Section 4. The objective of the rat *in vivo* studies with the individual food additives was to determine suitable dietary levels of the compounds for subsequent mixtures studies and to identify suitable biomarkers of effect of the compounds for use in the subsequent mixtures studies.

Each of the four test compounds was administered in the diet to male Sprague-Dawley rats for 28 days at five dose levels. In addition, the middle and highest (i.e. the third and fifth highest, respectively) dose levels were also administered for a period of 3 days. The purpose of the limited 3 day *in vivo* studies was to obtain some additional information on the short term effects of the food additives to compare with the subsequent 3 day *in vitro* studies (see Sections 6.6., 6.7., 6.9. and 6.10.).

The five dose levels of each of the four food additives were selected from an evaluation of published literature and other data available to the ILSI-Europe Acceptable Daily Intake Task Force. The target dose levels as mg/kg/day doses are shown below in Table 2. In order to convert these target mg/kg/day dose levels into dietary concentrations (i.e. ppm in the diet), use was made of body weight gain and food consumption data from a number of previous fully GLP compliant 28 day studies conducted at BIBRA with male Sprague-Dawley rats. Based on this data, typical body weights of six (i.e. day 0) and ten (i.e. day 28) week old male Sprague-Dawley rats were calculated to be 158 and 287.5 g, respectively, with a mean body weight of 231 g throughout the study. From food consumption data, a mean value of 22.75 g diet/day was selected. The required dietary levels of the food additives were thus calculated from the equation.

$$\text{Compound dietary level (mg/g diet)} = \text{Desired intake (mg/kg/day)} \times \frac{231}{22.75 \times 1000}$$

$$= \text{Desired intake} \times 0.010153846$$

The calculated five dietary levels (in units of ppm in the diet) for each of the four food additives are shown in Table 3.

Table 2. Target and actual mean daily intakes for male Sprague-Dawley rats fed various dietary levels of BHT, CC, PG and TB for 28 days

Group	Compound	Target daily intake (mg/kg/day)[a]	Actual daily intake (mg/kg/day)[b]	Percentage of target daily intake
A	Control	–	–	–
B	BHT	25	27.8	111
C	BHT	75	87.8	117
D	BHT	150	166.9	111
E	BHT	300	321.4	107
F	BHT	1000	1158.8	116
G	CC	25	26.4	106
H	CC	75	84.8	113
I	CC	200	224.8	112
J	CC	400	459.7	115
K	CC	1000	1117.8	112
L	PG	20	20.4	102
M	PG	50	49.9	100
N	PG	150	160.0	107
O	PG	300	359.6	120
P	PG	600	658.4	110
Q	TB	10	11.4	114
R	TB	25	29.6	118
S	TB	75	87.4	117
T	TB	200	200.6	100
U	TB	500	452.4	91

[a]Dose levels selected from literature and other available data.

[b]Calculated from body weight and food consumption data performed over study days 0–3, 3-7, 7-10, 10-14, 14-17, 17-21, 21-24 and 24-28.

6.1.2. Analysis of food additives in rodent diet

A high performance liquid chromatography (HPLC) method was developed for the simultaneous determination of levels of BHT, CC, PG and TB in rodent diet. At the

request of FSA, an additional objective of the rodent diet assay development was to confirm that standard rodent maintenance diet (i.e. R and M No.1 fine ground diet obtained from Special Diets Services, Witham, Essex) did not contain any appreciable levels of synthetic antioxidants (i.e. BHT and PG). The diet manufacturer confirmed that while rodent diet contains natural antioxidants (e.g. vitamins C and E), synthetic antioxidants were not directly added. For the study with the individual food additives a single batch (batch number 1454) of R and M No.1 fine ground diet was used. For each of the four food additives six samples from the lowest and highest and two samples from the other three dietary levels were analysed. The mean dietary concentration for each level of the four food additives are shown in Table 3. Overall, there was a good agreement between the target dietary levels and the actual analysed dietary levels, with percentage of nominal values ranging from 94–100%, 95–103%, 89–108% and 98–102% for BHT, CC, PG and TB, respectively (Table 3).

The methanol extracts of control (i.e. no test compound added) diet contained UV absorbing material with similar retention times to BHT, CC, PG and TB. The "apparent" levels of BHT and PG were around 9.8 and 1.3 ppm, respectively. It should be emphasised that these are only "apparent" levels in that they refer to UV absorbing material present in rodent diet extracts which elutes at the same retention times as authentic BHT and PG. While no attempt was made to identify this UV absorbing material (e.g. by liquid chromatography-mass spectrometry-mass spectrometry), their chromatographic behaviour suggested that these peaks contained UV absorbing material other than BHT and PG. In this study the lowest dietary levels of BHT and PG were 254 and 203 ppm, respectively. Hence, the "apparent" dietary levels of BHT and PG were only 3.9 and 0.6%, respectively, of the lowest dietary levels and hence were not considered to be of any toxicological significance for this study.

6.1.3. Compound intake and effect on body weight and liver weight

Male Sprague-Dawley rats were fed either control diet (n=ten per time point) or diets containing BHT, CC, PG and TB (n=six per dietary level and time point) for periods of 3 and 28 days. Animal body weight and food consumption data was monitored throughout the study and this data used to calculate mean daily intakes for each dietary level of the four food additives. Mean daily intakes of BHT, CC, PG and TB ranged from

27.8–1158.8, 26.4–1117.8, 20.4–658.4 and 11.4–452.4 mg/kg/day, respectively (Table 2). Overall, there was a good agreement between the target daily intakes and the actual achieved daily intakes of the four food additives. Percentage of target daily intake levels ranged from 107–117%, 106–115%, 100–120% and 91–118% for BHT, CC, PG and TB, respectively (Table 2). No animals were lost during the course of this study. With the exception of high doses of TB (see below) all the study diets were well tolerated by the animals.

In the limited 3 day studies, rats were fed control diet or diets containing 1523 and 10154 ppm BHT (dietary levels refer to target levels), 2031 and 10154 ppm CC, 1523 and 6092 ppm PG and 762 and 5077 ppm TB. While treatment with 1523 ppm BHT and 2031 and 10154 ppm CC had no significant effect on body weight, significant decreases were observed in rats given 10154 ppm BHT and both dose levels of PG and TB (Table 4). In rats fed 1523 and 10154 ppm BHT absolute liver weights were significantly increased to 116 and 131% of control, respectively, and relative liver weights were increased to 117 and 149% of control, respectively (Table 4). While treatment with CC, PG and TB had no significant effect on relative liver weight, significant decreases in absolute liver weight were observed in rats given 1523 ppm PG and 5077 ppm TB (Table 4).

The effect of 28 days treatment with BHT, CC, PG and TB on body weight, liver weight and relative liver weight is shown below in Table 5. Terminal body weight was significantly reduced in rats given the highest dietary levels of BHT and CC and four of the five dietary levels of PG. A significant reduction in body weight was also observed in rats given 2031 and 5077 ppm TB (Table 5). While treatment with 102-762 ppm TB had little effect on food consumption (data not shown), the administration of 2031 and 5077 ppm TB reduced food consumption to 75 and 54% of control, respectively. The reduction in body weight in rats given high doses of TB may be at least partially attributable to the palatability of the diets.

The treatment of rats with 1523-10154 ppm BHT for 28 days significantly increased absolute liver weight, whereas significant decreases were observed at some dose levels of PG and TB (Table 5). While CC and PG had no effect on relative liver weight,

significant dose-dependent increases in relative liver weight were observed in rats given 762-10154 ppm BHT and 762-5077 ppm TB (Table 5). At the highest dose levels of BHT and TB examined, relative liver weight was increased to 155 and 141% of control, respectively.

Table 5. Effect of treatment of rats for 28 days with BHT, CC, PG and TB on body weight, liver weight and relative liver weight

Group	Treatment (ppm)[a]	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
A	Control	280.8 ± 0.6 (100)[b]	10.8 ± 0.6 (100)	3.85 ± 0.21 (100)
B	BHT 254	277.8 ± 17.8 (99)	11.0 ± 1.5 (102)	3.95 ± 0.36 (103)
C	BHT 762	275.2 ± 13.9 (98)	11.6 ± 0.6 (108)	4.23 ± 0.22 (110)**
D	BHT 1523	278.0 ± 14.0 (99)	13.0 ± 0.9 (120)***	4.66 ± 0.15 (121)***
E	BHT 3046	279.3 ± 17.5 (100)	13.9 ± 0.8 (129)***	4.97 ± 0.17 (129)***
F	BHT 10154	236.5 ± 12.6 (84)***	14.1 ± 0.8 (131)***	5.97 ± 0.38 (155)***
G	CC 254	281.7 ± 8.8 (100)	10.3 ± 1.2 (96)	3.66 ± 0.10 (95)
H	CC 762	282.5 ± 15.0 (101)	10.8 ± 0.6 (100)	3.82 ± 0.14 (99)
I	CC 2031	276.0 ± 19.3 (98)	10.6 ± 0.8 (99)	3.86 ± 0.18 (100)
J	CC 4062	266.7 ± 15.0 (95)	10.0 ± 0.8 (92)	3.74 ± 0.20 (97)
K	CC 10154	263.5 ± 13.3 (94)*	10.1 ± 1.0 (94)	3.84 ± 0.28 (100)
L	PG 203	255.0 ± 15.6 (91)**	9.6 ± 1.2 (89)*	3.77 ± 0.36 (98)
M	PG 508	253.0 ± 17.8 (90)**	9.3 ± 1.1 (86)**	3.65 ± 0.20 (95)
N	PG 1523	234.2 ± 14.5 (83)***	8.8 ± 1.0 (82)***	3.76 ± 0.25 (98)
O	PG 3046	275.2 ± 20.7 (98)	10.7 ± 0.9 (99)	3.89 ± 0.11 (101)
P	PG 6092	255.5 ± 13.8 (91)**	10.4 ± 0.8 (97)	4.09 ± 0.19 (106)
Q	TB 102	272.7 ± 28.9 (97)	10.5 ± 1.0 (97)	3.86 ± 0.25 (100)
R	TB 254	269.2 ± 25.5 (96)	10.5 ± 1.8 (97)	3.87 ± 0.34 (101)
S	TB 762	271.2 ± 17.3 (97)	11.2 ± 0.9 (104)	4.14 ± 0.16 (108)*
T	TB 2031	229.3 ± 13.7 (82)***	10.1 ± 0.7 (94)	4.42 ± 0.18 (115)***
U	TB 5077	170.7 ± 5.6 (61)***	9.2 ± 0.5 (86)**	5.42 ± 0.33 (141)***

[a]Dietary levels are target levels (ppm in diets).

[b]Results are presented as mean ± SD for groups of 10 control (group A) and 6 treated (groups B to U) rats. Percentage of control values are shown in parentheses.

Values significantly different from control are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Morphological examination of liver sections from control rats and from rats treated with BHT, CC, PG and TB was performed by TNO. Compared to control animals, no microscopic changes were observed in liver sections from rats treated with CC and PG. In the highest BHT group (10154 ppm), hepatocyte enlargement and nucleolar enlargement indicative of hepatocellular hypertrophy were observed. The treatment of rats with 5077 ppm TB resulted in decreased glycogen content and nucleolar enlargement.

6.1.4. Biochemical investigations

With the exception of one rat treated with 5077 ppm TB, serum samples were obtained from all control animals and from animals treated with BHT, CC, PG and TB for 28 days. These serum samples were analysed on the day of necropsy at BIBRA for levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). The remaining serum sample of each animal was frozen at –80°C. After transportation on dry ice to TNO, these samples were subsequently analysed for levels of sorbitol dehydrogenase (SDH), total protein, albumin, triglycerides and cholesterol.

The results of the serum analysis performed at BIBRA and TNO are shown in Tables 6 and 7, respectively. Generally, none of the compounds had any marked effect on serum ALT, AST and LDH activities (Table 6). Some increases in serum ALT and AST activities were observed in rats given 2031 ppm TB and 10154 ppm CC, respectively, whereas LDH activity was increased in rats given 3046 ppm BHT and 5077 ppm TB. The treatment of rats with 10154 ppm BHT, 762 and 2031 CC, 6092 ppm PG and 2031 and 5077 ppm TB produced significant increases in serum SDH activity (Table 7). While treatment with BHT, CC and PG had no effect on levels of total protein and albumin, significant increases were observed in rats given 2031 and 5077 ppm TB (Table 7). Serum triglyceride levels were significantly decreased in rats treated with 10154 ppm BHT and 4062 and 10154 ppm CC, whereas a significant increase in serum triglyceride levels was observed in rats given 6092 ppm PG (Table 7). Serum cholesterol levels were significantly decreased in rats given 2031-10154 ppm CC and 1523 and 6092 ppm PG, whereas significant increases in serum cholesterol levels were observed in rats given 3046 and 10154 ppm BHT and 2031 and 5077 ppm TB (Table 7).

Liver whole homogenate, washed microsomal and cytosolic fractions were prepared from all animals fed control diet and diets containing BHT, CC, PG and TB for 28 days. Washed microsomal and cytosolic fractions were assayed for protein content. Microsomal protein content was significantly increased in rats given the two highest dose levels of BHT (Table 8). Small increases were also observed in rats given 254 ppm CC and 3046 ppm PG. Cytosolic protein content was significantly increased in rats given the two highest dose levels of PG and in rats given 254 ppm CC and 5077 ppm TB (Table 8). In contrast, treatment with 762-10154 ppm BHT resulted in significant decreases in cytosolic protein content. To allow for the effects of the food additives on microsomal and cytosolic protein content, the CYP and GST activities measured were expressed per gram of liver, rather than per unit of either microsomal or cytosolic protein.

Liver microsomal fractions were assayed for total CYP content and for 7-ethoxyresorufin O-deethylase and 7-pentoxyresorufin O-dependentylase activities. Although 7-ethoxyresorufin is metabolised mainly by CYP2C6 in liver microsomes from untreated rats and 7-pentoxyresorufin is metabolised by several CYP forms, 7-ethoxyresorufin and 7-pentoxyresorufin are considered good markers for induction of CYP1A and CYP2B forms, respectively (Burke et al., 1985, 1994; Nerurkar et al., 1993; Nims and Lubet, 1996). The treatment of rats with the two highest dose levels of BHT resulted in significant increases in microsomal total CYP content (Table 9). While total CYP content was not affected by treatment with CC, small decreases were observed in rats treated with 1523 ppm PG and 254 ppm TB.

Treatment with 762-10154 ppm BHT increased hepatic microsomal 7-ethoxyresorufin O-deethylase activity to 171-193% of control (Table 9). A small increase in enzyme activity was also observed in rats given 254 ppm CC, whereas treatment with 762-5077 ppm TB produced a dose-dependent increase in 7-ethoxyresorufin O-deethylase activity up to 544% of control (Table 9).

Table 9. Effect of treatment of rats for 28 days with BHT, CC, PG and TB on hepatic microsomal CYP content and 7-ethoxyresorufin O-deethylase and 7-pentoxyresorufin O-dependentylase activities

Group	Treatment (ppm)[a]	CYP content (nmol/g liver)	7-Ethoxyresorufin O-deethylase (nmol/min/g liver)	7-Pentoxyresorufin O-dependentylase (nmol/min/g liver)
A	Control	29.7 ± 0.9 (100)[b]	1.01 ± 0.07 (100)	0.46 ± 0.03 (100)
B	BHT 254	28.9 ± 0.7 (97)	1.23 ± 0.14 (121)	0.80 ± 0.10 (172)***
C	BHT 762	30.3 ± 2.3 (102)	1.79 ± 0.24 (177)***	4.87 ± 0.66 (1045)***
D	BHT 1523	32.2 ± 1.3 (109)	1.73 ± 0.10 (171)***	16.85 ± 1.73 (3630)***
E	BHT 3046	43.8 ± 2.5 (147)***	1.77 ± 0.19 (175)***	50.44 ± 2.46 (10860)***
F	BHT 10154	42.3 ± 1.1 (142)***	1.96 ± 0.16 (193)***	57.66 ± 2.92 (12410)***
G	CC 254	29.7 ± 1.6 (100)	1.26 ± 0.08 (125)*	0.42 ± 0.02 (91)
H	CC 762	31.1 ± 1.9 (105)	1.10 ± 0.08 (109)	0.42 ± 0.02 (91)
I	CC 2031	28.7 ± 1.3 (97)	1.05 ± 0.08 (103)	0.45 ± 0.01 (96)
J	CC 4062	29.2 ± 1.3 (98)	1.01 ± 0.11 (100)	0.46 ± 0.03 (100)
K	CC 10154	30.3 ± 1.1 (102)	1.09 ± 0.04 (108)	0.56 ± 0.04 (120)*
L	PG 203	28.3 ± 2.2 (95)	0.93 ± 0.05 (91)	2.35 ± 0.49 (505)***
M	PG 508	26.4 ± 0.3 (89)	0.85 ± 0.06 (84)	0.86 ± 0.20 (185)**
N	PG 1523	24.4 ± 2.3 (82)*	0.86 ± 0.09 (85)	0.49 ± 0.03 (105)
O	PG 3046	30.3 ± 1.4 (102)	0.95 ± 0.10 (94)	0.51 ± 0.02 (109)
P	PG 6092	31.1 ± 1.4 (105)	1.04 ± 0.09 (103)	0.54 ± 0.03 (116)
Q	TB 102	28.8 ± 1.3 (97)	1.10 ± 0.06 (109)	0.43 ± 0.02 (92)
R	TB 254	26.3 ± 1.5 (89)*	1.09 ± 0.08 (108)	0.46 ± 0.02 (98)
S	TB 762	30.5 ± 1.0 (103)	1.84 ± 0.09 (181)***	0.53 ± 0.01 (115)
T	TB 2031	29.7 ± 1.3 (100)	2.76 ± 0.16 (273)***	1.01 ± 0.04 (217)***
U	TB 5077	30.6 ± 1.2 (103)	5.51 ± 0.40 (544)***	3.15 ± 0.56 (677)***

[a]Dietary levels are target levels (ppm in diets).

[b]Results are presented as mean ± SEM for groups of 10 control (group A) and 6 treated (groups B to U) rats. Percentage of control values are shown in parentheses.

Values significantly different from control are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Treatment with 254-10154 ppm BHT produced a marked dose-dependent increase in hepatic microsomal 7-pentoxyresorufin O-dependentylase activity (Table 9). Enzyme activity was increased to 12410% of control by treatment with 10154 ppm BHT, whereas

7-pentoxyresorufin O-depentylase activity was increased to 217 and 677% of control by treatment with 2031 and 5077 ppm TB, respectively. Treatment with the highest dose of CC also resulted in a small increase in 7-pentoxyresorufin O-depentylase activity. Enzyme activity was also significantly increased by treatment with 203 and 508 ppm PG, but not by higher dietary levels of PG (Table 9).

Western immunoblotting studies were performed with liver microsomes from control rats and from rats given the mid and the highest dose levels of the food additives. These dose levels comprised 1523 and 10154 ppm for BHT, 2031 and 10154 ppm for CC, 1523 and 6092 ppm for PG and 762 and 5077 ppm for TB. Studies were performed with antipeptide antibodies to rat CYP1A1, CYP1A2, and CYP2B1/2 (i.e. to CYP2B1 and CYP2B2). The specificity of these antibodies has been previously described (Edwards, 1998).

Representative Western blots showing the effects observed following with the food additives have been published (Price et al., 2004; Stierum et al., 2008). As would be expected (Edwards, 1998), CYP1A1 apoprotein was not detected in liver microsomes from control rats even after long exposure times. Moreover, no CYP1A1 apoprotein was detected with liver microsomes from rats treated with mid and high doses of BHT, CC, PG and TB. The ability of the antibody used to detect CYP1A1 apoprotein was confirmed by the use of liver microsomes from rats treated with β-naphthoflavone, which is know to induce CYP1A1 in the rat (Okey, 1990). Unlike CYP1A1, levels of CYP1A2 apoprotein were readily detectable in liver microsomes from control rats and from rats treated with BHT, CC, PG and TB. Treatment with TB, but not BHT, CC and PG, clearly increased levels of CYP1A2 apoprotein.

The CYP2B1/2 antibody recognised two immunoreactive bands. Using long exposure times these bands were detectable in liver microsomes from control rats and from rats treated with BHT, CC, PG and TB. Previous studies have demonstrated that the amino acid sequences CYP2B1 and CYP2B2 are >97% identical with both CYP forms having similar molecular weights (Nims and Lubet, 1996). Hence the two immunoreactive bands detected with the CYP2B1/2 antibody constitute CYP2B1 and CYP2B2. Treatment with 1523 and 10154 ppm BHT markedly induced levels of CYP2B1/2 apoprotein. Levels of

CYP2B1/2 apoprotein were also increased by TB, particularly at the highest dose level examined.

Total RNA was extracted from liver samples from control rats and from rats treated with BHT, CC, PG and TB. Levels of CYP2B1 and CYP2B1/2 (i.e. CYP2B1 and CYP2B2) mRNAs were determined by real-time quantitative RT-PCR methodology (TaqMan®). To normalise for RNA loading, levels of CYP2B1 and CYP2B1/2 mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction. Treatment with 254-10154 ppm BHT resulted in a marked dose-dependent induction in CYP2B1 and CYP2B1/2 mRNA levels (Table 10). Significant increases in CYP2B1 and CYP2B1/2 mRNA levels were also observed after treatment with 2031 and 5077 ppm TB. Treatment with 254-10154 ppm CC also resulted in some increases in CYP2B1 mRNA levels, with CYP2B1/2 mRNA levels also being increased at dose levels of 4062 and 10154 ppm CC (Table 10). In contrast, only treatment with 6092 ppm PG resulted in an increase in CYP2B1 mRNA levels.

As TB is known to induce CYP1A forms in rat and human hepatoma cell lines and in cultured rabbit hepatocytes (Groten et al., 2000), levels of CYP1A1 and CYP1A2 mRNA were determined in liver samples from control rats and from rats given 102-5077 ppm TB in the diet for 28 days. For purposes of comparison, CYP1A1 and CYP1A2 mRNA levels were also determined in liver samples from rats given 1523 and 10154 ppm BHT in the diet for 28 days. The treatment of rats with 102-5077 ppm TB produced a dose-dependent induction of hepatic CYP1A1 (Fig. 1A) and CYP1A2 (Fig. 1B) mRNA levels. While treatment with 102-762 ppm TB produced only small increases (≤5-fold) in CYP1A1 and CYP1A2 mRNA levels, treatment with 2031 and 5077 ppm TB produced 36- and 169-fold increases, respectively, in CYP1A1 mRNA levels and 8- and 25-fold increases, respectively, in CYP1A2 mRNA levels. In contrast, treatment with 1523 and 10154 ppm BHT produced only small increases (≤4-fold) in rat hepatic CYP1A1 (Fig. 1A) and CYP1A2 (Fig. 1B) mRNA levels.

The effect of treatment with the four food additives on some rat hepatic cytosolic GST activities was also determined. Two distinct GST superfamilies are known to exist, namely the membrane-associated (microsomal) and soluble (cytosolic) GST

superfamilies (Sherratt and Hayes, 2002). On the basis of their degree of sequence identity, the soluble mammalian enzymes have been assigned to eight families or classes, which include the alpha, mu, pi and theta classes (Beckett and Hayes, 1993; Eaton and Brammler, 1999; Mannervik et al., 1985). Soluble GST forms exist as dimeric proteins, with subunit molecular weights of around 25 kDa.

A variety of substrates are available to assay GST activities. While some substrates, such as CDNB, are metabolised by many GST forms, other substrates are considered useful for studying particular GST class enzyme activities. For example, CH is considered a useful substrate for alpha class forms, DCNB and TPBO for mu class forms, EA for pi class forms and ENPP for theta class forms (Beckett and Hayes, 1993; Eaton and Brammler, 1999; Mannervik et al., 1985). To examine the induction of GSTs by BHT, CC, PG and TB, enzyme activities were determined employing CDNB, CH, DCNB, EA and ENPP as substrates. In addition, the effect of CC and PG on GST activity towards TPBO as substrate was also determined.

Treatment with BHT resulted in dose-dependent increases in GST activity towards several of the substrates examined (Tables 11 and 12). GST activity towards CDNB as substrate was significantly increased by treatment with 762-10154 ppm BHT (Table 11), whereas GST activity towards DCNB, EA and ENPP as substrates was significantly increased by treatment with 1523-10154 ppm BHT (Tables 11 and 12). In addition, GST activity towards CH as substrate was significantly increased by treatment with 10154 ppm BHT (Table 11).

Like BHT, treatment with TB also resulted in dose-dependent increases in GST activities. GST activity towards DCNB as substrate was significantly increased by treatment with 102-5077 ppm TB, whereas GST activity towards EA and ENPP as substrates was significantly increased by treatment with 762-5077 and 254-5077 ppm TB, respectively (Tables 11 and 12). In addition, GST activity towards CDNB and CH as substrates was significantly increased by treatment with 2031 and 5077 ppm TB (Table 11).

Table 11. Effect of treatment of rats for 28 days with BHT, CC, PG and TB on hepatic cytosolic GST activities towards CDNB, CH and DCNB as substrates

Group	Treatment (ppm)[a]	GST activity (μmol/min/g liver)[b]		
		Substrate CDNB	Substrate CH	Substrate DCNB
A	Control	167.1 ± 10.8 (100)[c]	63.94 ± 3.95 (100)	10.46 ± 0.30 (100)
B	BHT 254	198.5 ± 15.5 (119)	63.77 ± 2.43 (100)	10.38 ± 0.62 (99)
C	BHT 762	235.0 ± 15.1 (141)**	65.00 ± 1.99 (102)	11.47 ± 0.83 (110)
D	BHT 1523	286.0 ± 12.2 (171)***	60.48 ± 3.81 (95)	14.54 ± 1.34 (139)***
E	BHT 3046	312.4 ± 11.0 (187)***	72.83 ± 3.49 (114)	19.83 ± 1.00 (190)***
F	BHT 10154	650.1 ± 34.1 (389)***	78.07 ± 3.39 (122)**	26.49 ± 1.31 (253)***
G	CC 254	206.7 ± 21.8 (124)	64.55 ± 6.07 (101)	10.58 ± 0.49 (101)
H	CC 762	180.6 ± 13.0 (108)	68.72 ± 3.84 (107)	11.29 ± 0.64 (108)
I	CC 2031	166.9 ± 21.9 (100)	75.82 ± 7.34 (119)	11.65 ± 0.96 (111)
J	CC 4062	189.9 ± 19.0 (114)	67.55 ± 2.04 (106)	10.46 ± 0.42 (100)
K	CC 10154	167.1 ± 16.8 (100)	69.01 ± 3.46 (108)	8.97 ± 0.32 (86)*
L	PG 203	217.6 ± 12.0 (130)*	71.37 ± 3.27 (112)	9.87 ± 0.47 (94)
M	PG 508	183.1 ± 22.0 (110)	65.88 ± 1.48 (103)	9.26 ± 0.49 (89)
N	PG 1523	180.9 ± 16.1 (108)	64.30 ± 3.39 (101)	9.23 ± 0.39 (88)
O	PG 3046	214.0 ± 9.1 (128)*	63.49 ± 6.29 (99)	11.24 ± 0.60 (107)
P	PG 6092	209.1 ± 13.3 (125)	65.92 ± 3.76 (103)	10.94 ± 0.82 (105)
Q	TB 102	207.8 ± 15.8 (124)	75.24 ± 2.21 (118)	11.90 ± 0.44 (114)*
R	TB 254	204.7 ± 17.2 (123)	71.26 ± 3.72 (111)	13.56 ± 0.73 (130)***
S	TB 762	175.1 ± 24.2 (105)	69.73 ± 1.96 (109)	12.91 ± 0.56 (123)***
T	TB 2031	348.4 ± 41.7 (208)***	77.87 ± 5.80 (122)*	15.32 ± 0.77 (146)***
U	TB 5077	522.0 ± 58.9 (312)***	92.82 ± 5.66 (145)***	18.52 ± 0.65 (177)***

[a]Dietary levels are target levels (ppm in diets).

[b]GST substrates were: CDNB, 1-chloro-2,4-dinitrobenzene; CH, cumene hydroperoxide; DCNB, 1,2-dichloro-4-nitrobenzene.

[c]Results are presented as mean ± SEM for groups of 10 control (group A) and 6 treated (groups B to U) rats. Percentage of control values are shown in parentheses.

Values significantly different from control are: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Unlike BHT and TB, both CC and PG had less marked effects on GST activity towards the substrates studied. Treatment with CC had no significant effect on GST activity

towards CDNB and CH as substrates, whereas treatment with PG had no significant effect on GST activity towards CH, DCNB, TPBO and EA as substrates (Tables11 and 12). While small increases in GST activity towards TPBO and EA as substrates were observed at some dose levels of CC, GST activity towards DCNB and ENPP as substrates was significantly reduced in rats given 10154 ppm CC (Tables 11 and 12). Treatment with 203 and 3046 ppm PG significantly increased GST activity towards CDNB as substrate, whereas GST activity towards ENPP as substrate was increased after treatment with 6092 ppm PG.

The effect of treatment with the food additives on rat hepatic GSTP1 and GSTT1 mRNA levels was also studied as TaqMan® primers and probe sets were available for these two GST forms. Treatment with all four food additives produced significant increases in GSTP1 mRNA levels. While 254-3046 ppm BHT had no significant effect, treatment with 10154 ppm BHT produced a 102-fold increase in GSTP1 mRNA levels (Table 13). Treatment with CC produced increases in GSTP1 mRNA levels of 10-, 34- and 30-fold in rats fed diets containing 254, 762 and 2031 ppm CC, respectively, with no significant induction being observed at higher dietary levels of CC (Table 13). While 203 and 508 ppm PG had no effect on GSTP1 mRNA levels, treatment with 1523, 3046 and 6092 ppm PG produced 4-, 15- and 22-fold increases, respectively, in GSTP1 mRNA levels. GSTP1 mRNA levels were also increased 29- and 248-fold by treatment with 2031 and 5077 ppm TB (Table 13).

Treatment with the four food additives produced only small increases in hepatic GSTT1 mRNA levels. Small, but statistically significant, increases in hepatic GSTT1 mRNA levels were observed in rats given 1523-10154 ppm BHT, 254 ppm CC, 508 and 1523 ppm PG and 762-5077 ppm TB (Table 13). The greatest effect on GSTT1 mRNA levels was produced by 10154 ppm BHT, where a 4-fold increase was observed.

The transcriptomics data (see Section 6.2.) indicated that treatment with CC, but not BHT, PG and TB, resulted in expression of peroxisomal enoyl hydratase-like protein and CYP4A3. These results suggest that CC may be a rodent peroxisome proliferator and CYP4A form inducer (Ashby et al., 1994; Klaunig et al., 2003; Lake, 1995). In order to confirm the transcriptomics data, liver whole homogenates from control and CC treated

rats were assayed for cyanide-insensitive palmitoyl-CoA oxidation activity. Previous studies have demonstrated a good correlation between the induction of peroxisomal (palmitoyl-CoA) and microsomal (CYP4A-dependent) fatty acid oxidising enzyme activities (Lake and Lewis, 1996).

The effect of treatment with CC on hepatic palmitoyl-CoA oxidation activity is shown below in Table 14a. While treatment with 254-2031 ppm CC had no significant effect, hepatic palmitoyl-CoA oxidation activity was increased to 123 and 151% of control by treatment with 4062 and 10154 ppm CC, respectively (Table 14a). In light of the dose level employed this has to be considered as a weak effect.

Table 14a. Effect of treatment of rats for 28 days with CC on hepatic palmitoyl-CoA oxidation activity

Group	Treatment (ppm)[a]	Palmitoyl-CoA oxidation (µmol/min/g liver)
A	Control	0.71 ± 0.02 (100)[b]
G	CC 254	0.81 ± 0.03 (114)
H	CC 762	0.79 ± 0.03 (111)
I	CC 2031	0.78 ± 0.04 (110)
J	CC 4062	0.87 ± 0.02 (123)**
K	CC 10154	1.07 ± 0.04 (151)***

[a]Dietary levels are target levels (ppm in diets).

[b]Results are presented as mean ± SEM for groups of 6 rats. Percentage of control values are shown in parentheses.

Values significantly different from control are: ** $p<0.01$; *** $p<0.001$.

Trancriptome analysis for TB (see paragraph 6.2) demonstrated that 5 out of 12 genes from which the expression levels changed -including p53 itself- could be linked to p53 dependent pathways. In particular, increased GADD45 mRNA was observed. GADD45 is a well known downstream gene for p53 and transcriptionally upregulated upon p53 protein induction under conditions of oxidative stress (Kastan et al., 1992; Zhan et al.,

1994). Therefore, p53 protein levels were determined in whole liver homogenates, obtained from the 758 and 5188 ppm TB treatment groups. Table 14b shows p53 levels determined in selected TB dose groups, expressed both as pg/ml protein extract and ng/mg protein. Within this dose range tested, a modest dose-dependent increase up to 2-fold was observed which became highly significant ($p<0.001$) only in animals receiving the highest dose group of 5188 ppm TB. From a toxicological perspective this is a weak effect.

Table 14b. Nuclear oncoprotein p53 protein levels in livers obtained from TB-treated rats.

Treatment (ppm) [a]	p53 (pg /ml extract)	p53 (ng/mg protein)
Control	64.8 ± 19.5[b]	1.00 ± 0.18
TB 758	75.6 ± 21.0	1.50 ± 0.75
TB 5188	127.7 ± 42.4**	2.00 ± 0.58***

[a] Dietary levels are actual dietary levels (ppm in diets).

[b] Results are presented as mean ± standard deviation for 10 control and 6 treated rats, expressed both as pg p53 per ml tissue extract as well as ng of p53 per mg protein.

Values significantly different from control are: ** $p<0.01$; *** $p<0.001$

6.2. Transcriptomics Analysis of the Hepatic Effects of Individual Food Additives in the Rat

In short, RNA isolated from livers obtained from male Sprague Dawley rats treated for 28 days with additives was subjected to cDNA microarray analysis, employing Cy3 and Cy5 Dyes for gene expression signal detection in experimental samples, in relation to a common reference control RNA sample. Purpose was to identify possible markers of exposure or effect which could be employed in subsequent in vivo and in vitro studies to allow for interspecies extrapolation of possible mixture effects. Using a stringent filtering approach outlined in paragraph '5.7.1. Bioinformatics analysis performed on microarray data obtained from rat in vivo studies with individual food additives', the expression of 10 genes was found to change dose dependently upon treatment with BHT (Fig. 4). The hepatic mRNA levels of three cytochrome P450 forms increased: CYP2B1/2; CYP3A9

and CYP2C6. As well as induction of phase I xenobiotic metabolising enzymes, BHT was found to increase mRNA levels for glutathione S-transferase μ type 2, a phase II xenobiotic metabolizing enzyme. Also, the mRNA expression of carboxylesterase 10 precursor, probably also involved in drug metabolism, was increased. Miscellaneous genes which were found to change in a dose dependent manner include: interleukin-15; hematopoietic cell tyrosine kinase; zinc finger protein 179; tryptophan-2,3-dioxygenase and tropomyosin isoform 6.

Figure 4. Gene expression analysis range finding studies BHT

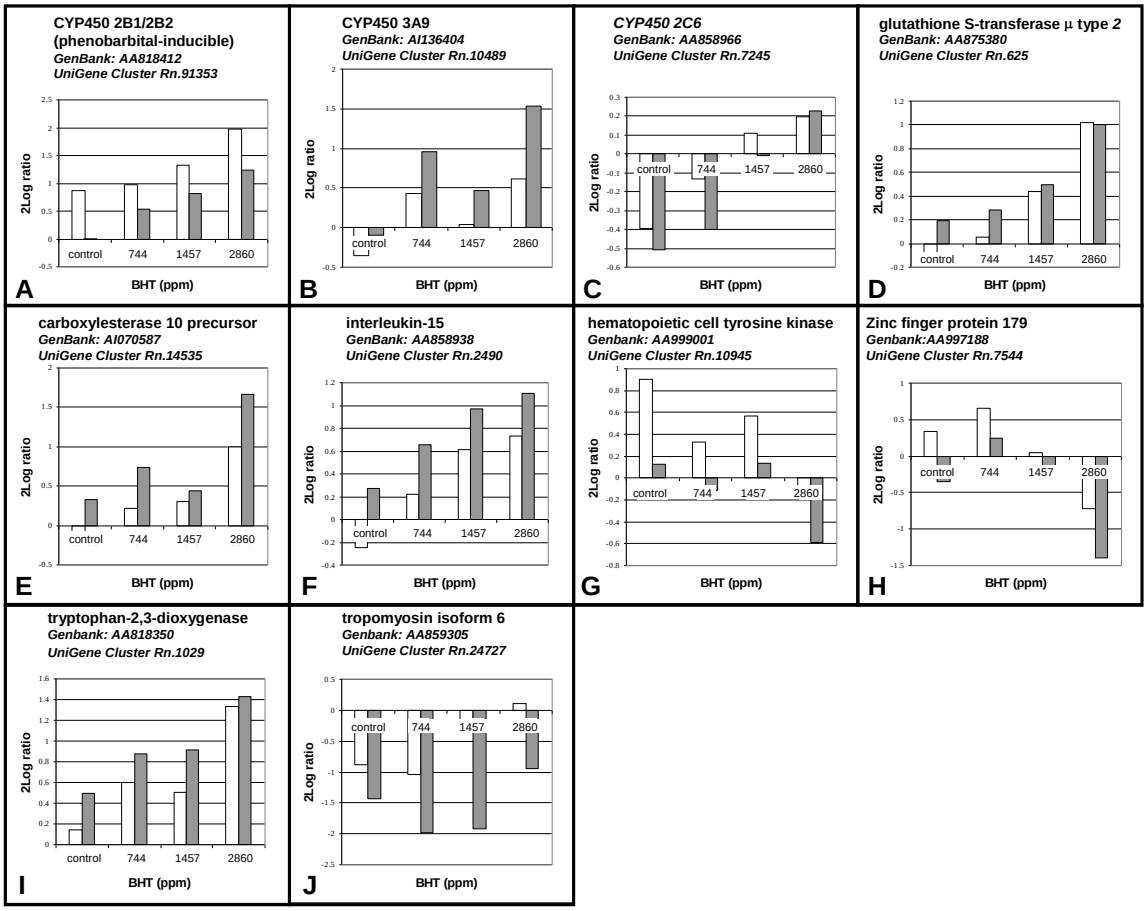

Figure 4A-4J. Genes differentially expressed, as determined by cDNA microarray analysis, upon oral administration of male Sprague-Dawley rats for 28 days to BHT. Shaded bars represent normalised, scaled 2log ratios of fluorescence signal obtained from Cy5 labelled RNA isolated from pooled liver samples from rats exposed to the food additive and fluorescence signal obtained from Cy3 labelled reference RNA. Open bars represent ratios from the dye swap experiment. Note that 4 out of 10 genes are involved in phase I and phase II xenobiotic metabolism (CYP2B1/2; CYP 3A9; CYP 2C6; glutathione S-transferase μ type

2. NOAEL and Effect Level for BHT in the rat are 25 and 100 mg/kg/day, respectively, corresponding to the intake of 254 and 1015 ppm diets in this study.

The hepatic expression levels of 12 genes changed upon administration of CC in the diet, as determined by the cDNA microarray analysis and data selection procedure employed here (Fig. 5). Two out of these 12 genes were peroxisomal, namely phytanoyl-CoA dioxygenase (decreased), enoyl-CoA hydratase-like protein (increased). Further, CYP4A3 (lauric acid omega hydroxylase 3) was upregulated, an enzyme known to be inducible by peroxisome proliferators (Lake and Lewis, 1996). Another CYP drug metabolising enzyme for which the mRNA levels were found to be increased was CYP2E1.

Figure 5. Gene expression analysis range finding studies CC

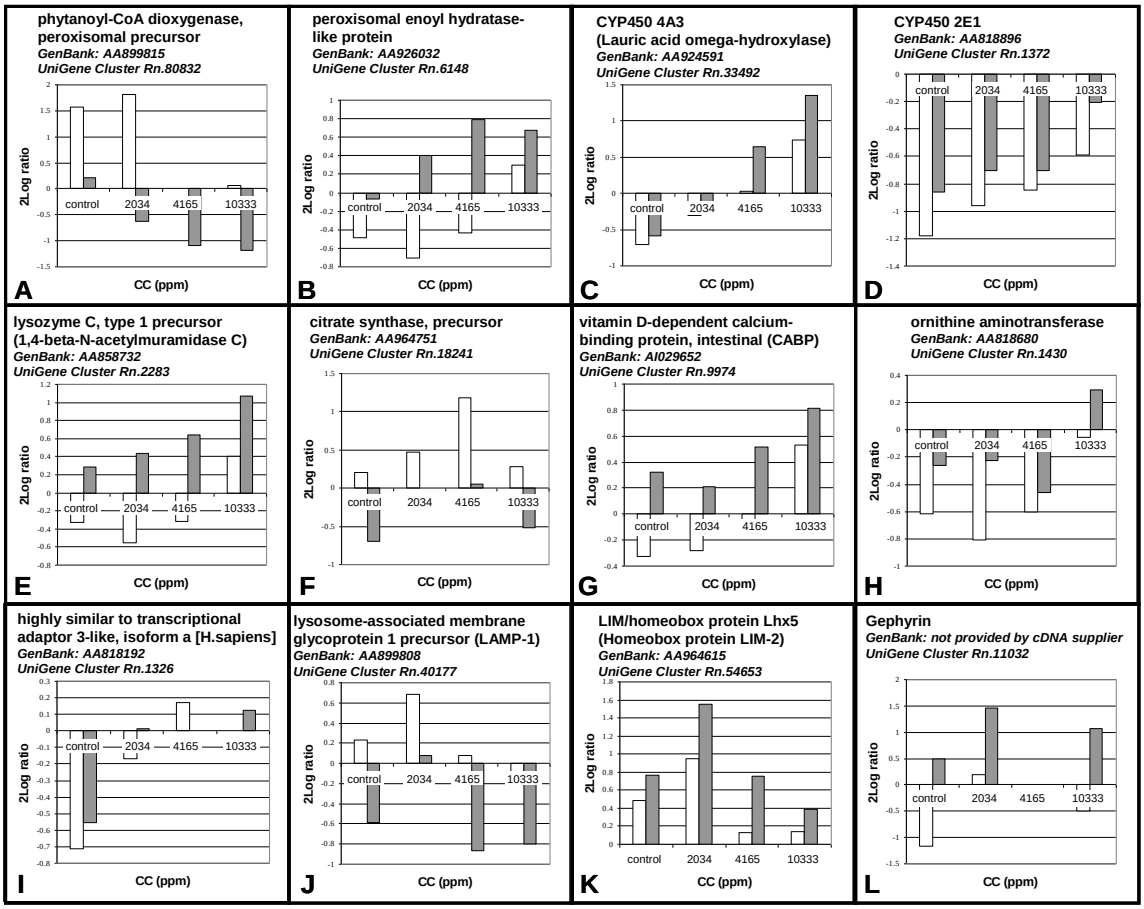

Figure 5A-5L. Genes differentially expressed, as determined by cDNA microarray analysis, upon oral administration of male Sprague-Dawley rats for 28 days to CC. Description of bars as in Fig. 4. Note that 3 out of 12 genes could be related to peroxisomal metabolism (phytanoyl-CoA dioxygenase; peroxisomal enoyl-CoA

hydratase-like protein; CYP 4A3). NOAEL and Effect Level for CC in the rat are 220 and 440 mg/kg/day, respectively, corresponding to the intake of 2234 and 4468 ppm diets in this study.

Other genes from which the expression level changed upon CC administration include: lysozyme C, type 1 precursor (1,4-beta-N-acetylmuramidase C); precursor sequence of citrate synthase; the intestinal vitamin D-dependent calcium-binding protein; ornithine aminotransferase; a rat cDNA which hybridised to a human sequence highly similar to transcriptional adaptor 3-like, isoform a; lysosome-associated membrane glycoprotein 1 precursor (LAMP-1); LIM/homeobox protein Lhx5 (Homeobox protein LIM-2) and gephyrin.

Administration of Sprague-Dawley rats for 28 days to PG (dose range 1464-6124 ppm) resulted in changes in the expression of eight genes (Fig. 6).

Figure 6. Gene expression analysis range finding studies PG

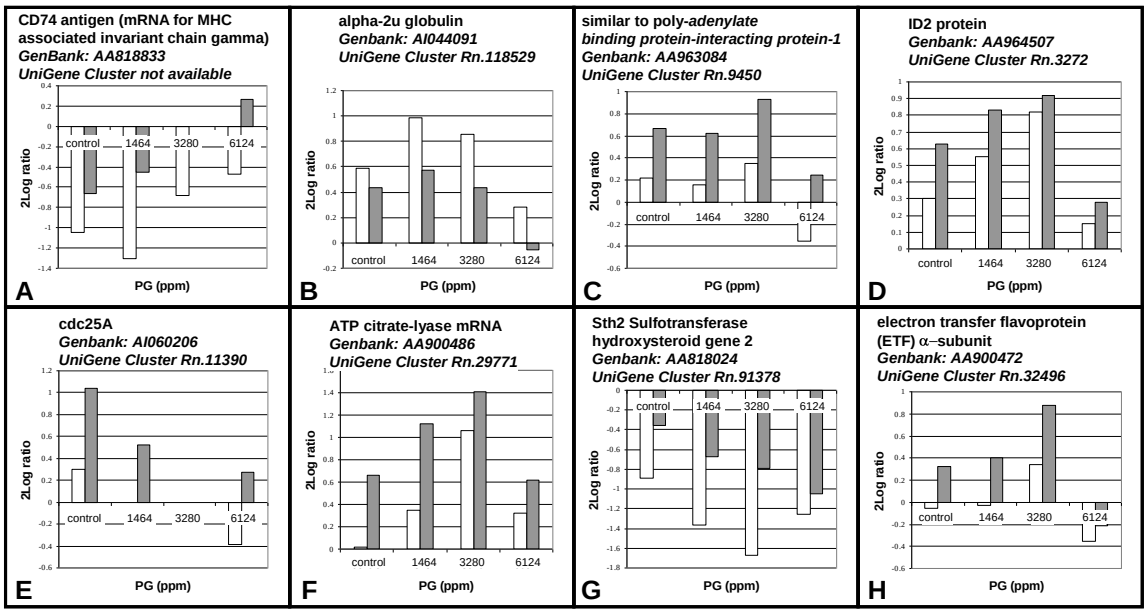

Figure 6A-6H. Genes differentially expressed, as determined by cDNA microarray analysis, upon oral administration of male Sprague-Dawley rats for 28 days to PG. Description of bars as in Fig. 4. NOAEL and Effect Level for PG in the rat are 135 and 527 mg/kg/day, respectively, corresponding to the intake of 1371 and 5351 ppm diets in this study.

The eight genes comprised CD74 antigen (invariant polpypeptide of major histocompatibility class II antigen-associated); submaxillary gland alpha-2u globulin; an EST moderately similar to *H. Sapiens* poly-adenylate binding protein-interacting protein-1; ID2 protein; cdc25A; ATP citrate-lyase mRNA; Sth2 Sulfotransferase hydroxysteroid gene 2 and electron transfer flavoprotein (ETF) α-subunit.

Microarray experiments indicated that TB administration resulted in dose-dependent changes in the expression of 12 genes (Fig. 7).

Figure 7. Gene expression analysis range finding studies TB

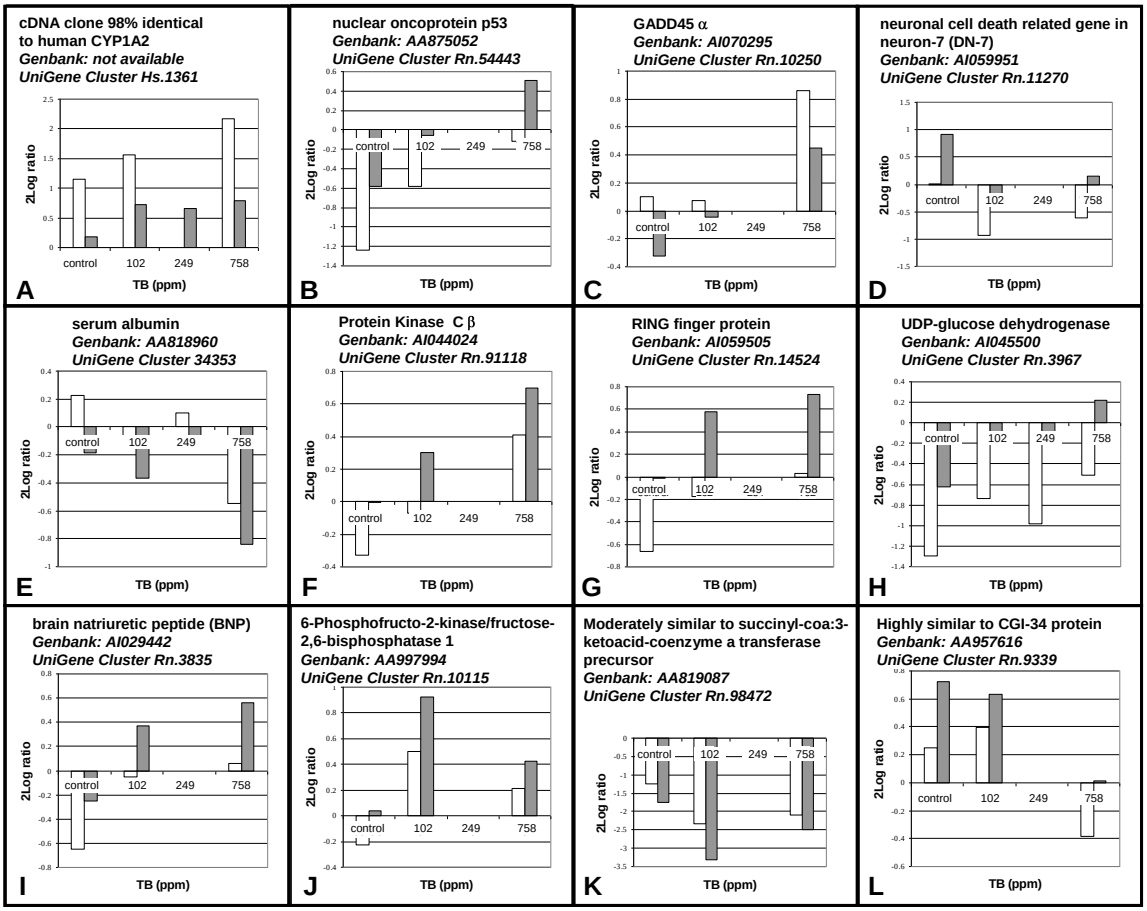

Figure 7A-7L. Genes differentially expressed, as determined by cDNA microarray analysis, upon oral administration of male Sprague-Dawley rats for 28 days to TB. Description of bars as in Fig. 4. Note the induction of CYP1A2, as well as changes in the expression of genes associated with p53 metabolism (nuclear oncoprotein p53; GADD45α; DN-7; serum albumin, protein kinase C β). NOAEL and Effect Level for TB in the rat are 10 and 37 mg/kg/day, respectively, corresponding to the intake of 102 and 376 ppm diets in this study.

The expression of a rat cDNA clone, 98% identical to the human CYP1A2 increased with TB treatment, with highest levels in the 758 ppm dose group. The expression of 5 genes associated with or under (partial) control of the nuclear oncoprotein p53 changed dose-dependently, in particular in the 758 ppm dose group, and include: p53 itself; growth arrest and damage inducible protein 45α (GADD45α); neuronal cell death related gene in neuron-7 (DN-7); serum albumin and protein kinase C β. Other genes which changed upon elevated dietary intake of TB were: RING finger protein; UDP-glucose dehydrogenase; brain natriuretic peptide (BNP); 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1; a cDNA moderately similar to a human precursor sequence for succinyl-coa:3-ketoacid-coenzyme A transferase and a cDNA highly similar to the human CGI-34 protein.

6.3. Rat in vivo Studies with Mixtures of Food Additives

6.3.1. Design of the rat in vivo mixtures study

The experimental design for the rat *in vivo* mixtures study with BHT, CC, PG and TB was provided by Professor John Groten and Dr Eric Schoen. Table 15a and Table 15b show the selected design with a control group (no compound treatment) and 27 experimental groups consisting of doses of one or more of the four food additives. For each of the four food additives the highest fractional dose level was designated 100 units, this being defined as a dose being around or somewhat higher than minimum observed adverse effect level, as observed in the range finding studies with individual additives It was necessary for the 100 unit dose levels to produce clear effects on some of the parameters measured, in order that effects, in terms of likelihood of interaction, if any, could also be observed at lower (e.g. 25 unit) dose levels.

From an examination of the data obtained from the previous *in vivo* dose-response study with the individual food additives, the following 100% fractional dose levels, corresponding to target or nominal dose levels were selected:

Food additive	Target dose (ppm in diet)
BHT	3046
CC	4062
PG	3046

TB	1750

These compound dose levels were selected on the basis of all the endpoints examined, including effects on body weight, liver weight and the various biomarkers measured (e.g. enzyme activities, mRNA levels), from the range finding studies with individual additives. The experimental design for the four compound mixtures study as shown in Table 15a and Table 15b comprised the following combinations:

Group 1	Control
Groups 2 to 13	Single compound exposures (25, 50 and 100 units or fractional dose levels))
Groups 14 to 19	Binary mixtures with a total dosage of 100 units
Group 20	A quaternary mixture with a total dosage of 100 units
Groups 21 to 26	Binary mixtures with a total dosage of 50 units
Group 27	A quaternary mixture with a total dosage of 50 units
Group 28	A quaternary mixture with a total dosage of 25 units

This 28 group design permitted the statistical determination for testing according to basic principles in mixture toxicology, which are to test if measured and predicted data are compatible with

- effect addition
- dose addition
- or interaction, being synergism (more than would expected based upon individual additives), or antagonism (less than would be expected based upon individual additives)

With emphasis on binary mixtures and some quaternary mixtures. The design is also further described in great detail in paragraph: 5.13.2. Statistical analysis of data from rat in vivo study with mixtures of food additives to infer mixtures effects, as well as in the document ‘Supplementary outline on statistical analysis, tables, results and guidance to supplementary supplementary electronic files’ (file: Supplementary statistics.doc).
(see Section 6.5.).

6.3.2. Analysis of food additives and food additive mixtures in rodent diet

A single batch of standard rodent maintenance diet (R and M No.1 fine ground diet) was used for the rat mixtures study. For each experimental group, a single 7 kg batch of diet was prepared and stored in closed containers at 4°C. The dietary levels of BHT, CC, PG and TB were analysed by the previously developed HPLC method (see Section 5.3.). This method permitted the simultaneous determination of levels of all four food additives and was sufficiently sensitive for the analysis of all of the required dietary concentrations of the test compounds.

Table 16 shows the target dietary levels and the actual analysed dietary levels of BHT, CC, PG and TB for all of the 27 experimental diets used in the mixtures study. The target dietary levels of 6.25, 12.5, 25 and 50 units of each compound were calculated from the selected 100 unit levels shown above. For example, for BHT the highest dietary level of 100 units comprised a dietary concentration of 3046 ppm, with dietary levels of 6.25, 12.5, 25 and 50 units corresponding to dietary concentrations of 190, 381, 762 and 1523 ppm, respectively (Table 16). Generally, there was a good agreement between the target dietary levels of the four food additives and the actual analysed concentrations in the study diets. For CC, PG and TB the analysed concentrations ranged from 96 to 106%, from 95 to 110% and from 102 to 106% of the target concentrations, respectively (Table 16). The analysed levels of BHT in the diets for all experimental groups, except group 28, ranged from 92 to 108% of the target concentrations. However, for group 28, which contained the lowest dietary level of BHT, the analysed concentration was 115% of the desired target level (Table 16).

6.3.3. Compound intake and effect on body weight and liver weight

Male Sprague-Dawley rats (around 6 weeks of age) were obtained from the same source as for the previous *in vivo* study with the individual food additives and were housed under the same experimental conditions. The animals were randomised into a control group of twelve animals and 27 experimental groups each containing six animals.

No animals were lost during the course of this study. Generally, all of the study diets were well tolerated by the animals. Animal body weight and food consumption was monitored through all 28 days of the study and this data used to calculate mean daily intakes of the food additives in each of the experimental diets. Table 17 shows the mean

compound intake data (expressed as mg/kg body weight/day) for each of the study diets. At the highest (i.e. 100 unit) dose levels, the mean daily intakes of BHT, CC, PG and TB were 333, 408, 290 and 153 mg/kg/day, respectively. Table 17 also contains the mean food consumption data (expressed as g diet/rat/day) for the control and experimental groups over the 28 day study period. With the exception of rats fed the highest dietary level of TB (target concentration 1750 ppm, actual concentration 1842 ppm), where a reduction in food consumption was noted, the food consumption of all the experimental groups was generally similar to that of the controls.

The effect of treatment of male Sprague-Dawley rats for 28 days with diets containing various combinations of BHT, CC, PG and TB on body weight is shown in Table 18. Terminal body weight was significantly reduced to 86% of control in rats fed the highest 1750 ppm dietary level of TB (dietary levels refer to target levels). This reduction in body weight gain was anticipated from the results of the previous rat *in vivo* range finding study with the selected food additives. A significant reduction in body weight to 87-91% of control was also observed in rats fed diets containing 1523 ppm BHT/875 ppm TB and 1523 ppm PG/875 ppm TB. Treatment of rats with a diet containing 762 ppm BHT/1016 ppm CC resulted in a small decrease in body weight to 94% of control (Table 18).

The treatment of rats for 28 days with diets containing various combinations of the four food additives produced significant effects on both absolute liver weight (Table 19) and relative liver weight (Table 20). In keeping with the effect on body weight, treatment with 1750 ppm TB resulted in a significant decrease in liver weight (Table 19). Examination of the data in Tables 19 and 20 (see below) reveals that treatment with the various study diets produced greater effects on relative liver weight, than on absolute liver weight. For example, the treatment of rats with 762, 1523 and 3046 ppm BHT produced significant dose-dependent increases in relative liver weight, whereas absolute liver weight was only significantly increased by treatment with 3046 ppm BHT. Apart from the effect of treatment with BHT alone, significant increases in relative liver weight to 109-121% of control were observed in rats fed diets containing BHT and one or more of the other food additives (Table 20). These diets comprised 1523 ppm BHT/2031 ppm CC, 1523 ppm BHT/1523 ppm PG, 1523 ppm BHT/875 ppm TB, 762 ppm

BHT/438 ppm TB, 762 ppm BHT/1016 ppm CC/762 ppm PG/438 ppm TB and 381 ppm BHT/508 ppm CC/381 ppm PG/219 ppm TB. Significant increases in relative liver weight were also observed in rats fed diets containing 438 ppm TB and 1016 ppm CC/438 ppm TB (Table 20).

Table 20. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on relative liver weight

Group	Treatment	Target compound concentration (ppm in diet)				Relative liver weight (g liver/100g body weight)[a]
		BHT	CC	PG	TB	
1	Control	-	-	-	-	3.72 ± 0.07 (100%)
2	BHT	762	-	-	-	4.12 ± 0.11 (111%)**
3	BHT	1523	-	-	-	4.31 ± 0.13 (116%)***
4	BHT	3046	-	-	-	4.91 ± 0.10 (132%)***
5	CC	-	1016	-	-	3.75 ± 0.16 (101%)
6	CC	-	2031	-	-	3.85 ± 0.09 (104%)
7	CC	-	4062	-	-	3.81 ± 0.10 (102%)
8	PG	-	-	762	-	3.71 ± 0.09 (100%)
9	PG	-	-	1523	-	3.72 ± 0.11 (100%)
10	PG	-	-	3046	-	3.52 ± 0.17 (95%)
11	TB	-	-	-	438	4.05 ± 0.15 (109%)*
12	TB	-	-	-	875	3.83 ± 0.13 (103%)
13	TB	-	-	-	1750	3.83 ± 0.07 (103%)
14	BHT/CC	1523	2031	-	-	4.45 ± 0.15 (120%)***
15	BHT/PG	1523	-	1523	-	4.36 ± 0.11 (117%)***
16	BHT/TB	1523	-	-	875	4.48 ± 0.21 (121%)***
17	CC/PG	-	2031	1523	-	3.84 ± 0.12 (103%)
18	CC/TB	-	2031	-	875	3.95 ± 0.08 (106%)
19	PG/TB	-	-	1523	875	4.00 ± 0.15 (108%)
20	BHT/CC/PG/TB	762	1016	762	438	4.21 ± 0.10 (113%)**
21	BHT/CC	762	1016	-	-	3.92 ± 0.07 (105%)
22	BHT/PG	762	-	762	-	4.00 ± 0.11 (108%)
23	BHT/TB	762	-	-	438	4.20 ± 0.11 (113%)**
24	CC/PG	-	1016	762	-	3.84 ± 0.11 (103%)
25	CC/TB	-	1016	-	438	4.02 ± 0.09 (108%)*
26	PG/TB	-	-	762	438	3.91 ± 0.13 (105%)

27	BHT/CC/PG/TB	381	508	381	219	4.05	± 0.13	(109%)*
28	BHT/CC/PG/TB	190	254	190	109	4.00	± 0.08	(108%)

[a]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats. Percentage of control values are shown in parentheses.
Values significantly different from control are: *p<0.05;**p<0.01;***p<0.001.

6.3.4. Biochemical investigations

The hepatic markers determined in the rat 28 day *in vivo* mixtures study comprised:

a). Microsomal and cytosolic protein content.

b). Microsomal total CYP content.

c). Microsomal 7-ethoxyresorufin O-deethylase and 7-pentoxyresorufin O-dependentylase activities.

d). Hepatic CYP1A2 and CYP2B1 mRNA levels.

e). Cytosolic GST activities towards CDNB and DCNB as substrates.

Toxicogenomics of single compounds revealed that gene expression proved to be sensitive in terms of detected compound exposure related effects. Two hypothesis generated from these experiment were pursued by further biochemical studies (peroxisome proliferation by CC and p53 induction by TB). From these it appeared that the effects were only modest, certainly in relation to the dose levels employed. Therefore these markers were not included in the following analysis of mixture effects in vivo and subsequent in vitro studies.

The effect of treatment of rats with BHT, CC, PG and TB either as single compound exposures or as binary and quaternary mixtures on hepatic microsomal protein content is shown in Table 21. Microsomal protein content was increased by treatment with some single compound dose levels of BHT and TB, but not with either CC or PG. The greatest increase in microsomal protein content to 136% of control was observed in rats given 3046 ppm BHT (Group 4). Increases in microsomal protein content were also observed after treatment with some of the binary and quaternary mixtures, in most instances these being in mixtures containing BHT and/or TB (Table 21).

Hepatic washed microsomal fractions were assayed for total CYP content and 7-ethoxyresorufin O-deethylase and 7-pentoxyresorufin O-depentylase activities. To take account of any changes in microsomal protein and relative liver weight, the CYP content and enzyme activity data has been calculated as specific activity or content per unit of microsomal protein, per gram of liver and per liver weight per kg body weight. When total CYP content was expressed as specific content (i.e. per unit of microsomal protein), significant increases in CYP content were only observed in a few treatment groups (Table 22). However, when CYP content was expressed per gram of liver and per relative liver weight per kg body weight, significant increases were observed in groups treated with single compound exposures of BHT and TB and with a number of the binary and quaternary mixtures of the food additives (Table 22). The greatest increase in total CYP content expressed per liver weight per kg body weight to 243% of control was observed in rats given 3046 ppm BHT (Group 4).

As shown in Table 23 below, the treatment of rats with all three single compound exposures of CC and PG had no significant effect on hepatic 7-ethoxyresorufin O-deethylase activity. In contrast, significant increases in specific 7-ethoxyresorufin O-deethylase activity (i.e. enzyme activity per unit of microsomal protein) were observed in rats given 3046 ppm BHT and 875 and 3046 ppm TB. For TB these increases in enzyme activity presumably represent the induction of CYP1A2, whereas for BHT the increases in enzyme activity are most likely due to the induction of CYP2C forms by this CYP2B inducer, rather than of CYP1A subfamily forms (Nims and Lubet, 1996; Okey, 1990; Price et al., 2004). In addition to the single compound exposures, significant increases in specific 7-ethoxyresorufin O-deethylase activity were observed with most of the binary mixtures containing TB and with two of the three quaternary mixtures studied (Table 23).

As shown in Table 24 below, treatment with BHT markedly induced hepatic microsomal 7-pentoxyresorufin O-depentylase activity, expressed as specific activity, per gram of liver or per liver weight per kg body weight. When 7-pentoxyresorufin O-depentylase activity was expressed as specific activity, per gram of liver and per liver weight per kg body weight treatment with 3046 ppm BHT (Group 4) increased enzyme activity to 7395, 9940 and 13,175% of control, respectively. Significant increases in 7-pentoxyresorufin O-depentylase activity were also observed in some of the single compound exposures of

CC, PG and TB (Table 24). 7-Pentoxyresorufin O-depentylase activity was also increased in rats given some of the binary and quaternary mixtures of the food additives, particularly with mixtures containing BHT.

Rat CYP mRNA levels were determined by real-time quantitative reverse transcription-polymerase chain reaction methodology (TaqMan®). Total RNA was extracted from all 174 liver samples from the *in vivo* mixtures study and reverse transcription performed (see Section 5.5.). The extraction of total RNA from 170 of these 174 liver samples resulted in cDNA preparations of adequate quality for subsequent TaqMan® analysis. However, for 4 liver samples cDNA preparations of inadequate quality were obtained and hence these animals (comprising three Group 1 controls and one animal from Group 8) were excluded from subsequent TaqMan® analysis of mRNA levels.

Table 23. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on hepatic microsomal 7-ethoxyresorufin O-deethylase activity

Group	Treatment[a]	7-Ethoxyresorufin O-deethylase[b]					
		(pmol/min/ mg protein)		(nmol/min/g liver)		(nmol/min/liver weight/ kg body weight)	
1	Control	17.3	± 1.03	0.64	± 0.043	23.6	± 1.61
2	BHT	22.4	± 2.74	0.87	± 0.103	35.5	± 3.78*
3	BHT	22.5	± 2.71	0.99	± 0.103**	43.0	± 5.42***
4	BHT	28.4	± 2.27***	1.40	± 0.112***	69.0	± 5.85***
5	CC	15.5	± 1.54	0.58	± 0.060	21.6	± 2.42
6	CC	19.8	± 0.85	0.69	± 0.066	26.4	± 3.15
7	CC	16.4	± 0.88	0.60	± 0.035	23.0	± 1.83
8	PG	19.0	± 2.12	0.69	± 0.094	25.7	± 3.84
9	PG	17.4	± 1.01	0.68	± 0.034	25.3	± 1.69
10	PG	13.0	± 0.62	0.49	± 0.015	17.1	± 1.20
11	TB	21.7	± 1.19	0.86	± 0.063	34.3	± 1.68
12	TB	36.2	± 1.00***	1.62	± 0.074***	62.3	± 3.66***
13	TB	52.9	± 3.91***	2.38	± 0.134***	91.4	± 6.16***
14	BHT/CC	18.0	± 1.19	0.83	± 0.060	37.0	± 2.87*
15	BHT/PG	17.8	± 1.24	0.80	± 0.038	34.9	± 1.64*

16	BHT/TB	32.7	± 2.77***	1.56	± 0.106***	69.5	± 5.44***
17	CC/PG	13.7	± 0.64	0.51	± 0.038	19.7	± 1.38
18	CC/TB	28.1	± 0.68***	1.27	± 0.032***	49.9	± 1.29***
19	PG/TB	29.9	± 1.17***	1.18	± 0.044***	47.2	± 3.03***
20	BHT/CC/PG/TB	28.9	± 3.44***	1.25	± 0.168***	53.0	± 8.07***
21	BHT/CC	20.4	± 3.56	0.82	± 0.167	32.4	± 6.91
22	BHT/PG	22.0	± 5.05	0.95	± 0.256*	37.6	± 9.76*
23	BHT/TB	32.7	± 4.98***	1.37	± 0.216***	57.7	± 9.17***
24	CC/PG	18.4	± 3.76	0.74	± 0.152	28.8	± 6.39
25	CC/TB	21.0	± 1.35	0.88	± 0.060	35.5	± 2.19*
26	PG/TB	24.5	± 1.11*	0.98	± 0.055**	38.3	± 1.78**
27	BHT/CC/PG/TB	23.3	± 1.58*	0.90	± 0.085*	36.2	± 3.15*
28	BHT/CC/PG/TB	16.5	± 1.19	0.60	± 0.044	23.9	± 1.73

[a]For details of the dietary levels of BHT, CC, PG and TB see Table 18.
[b]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats.
Values significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

Table 24. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on hepatic microsomal 7-pentoxyresorufin O-dependentylase activity

Group	Treatment[a]	7-Pentoxyresorufin O-dependentylase[b]					
		(pmol/min/ mg protein)		(nmol/min/g liver)		(nmol/min/liver weight/ kg body weight)	
1	Control	9.5	± 0.3	0.35	± 0.01	12.9	± 0.5
2	BHT	68.1	± 4.8***	2.66	± 0.26***	109.2	± 9.6***
3	BHT	235.5	± 26.3***	10.56	± 1.31***	459.0	± 66.0***
4	BHT	702.5	± 51.6***	34.79	± 2.71***	1699.4	± 109.2***
5	CC	54.5	± 13.8***	2.10	± 0.63***	78.8	± 23.6***
6	CC	11.1	± 0.8	0.41	± 0.03	16.0	± 1.4
7	CC	9.7	± 0.5	0.35	± 0.02	13.6	± 1.0
8	PG	14.1	± 0.6*	0.51	± 0.04*	18.9	± 1.2*
9	PG	16.9	± 2.3**	0.66	± 0.09***	24.6	± 3.4***
10	PG	13.6	± 1.7*	0.52	± 0.07*	18.2	± 2.9
11	TB	11.9	± 1.6	0.47	± 0.08	19.1	± 2.8*
12	TB	15.9	± 2.5	0.72	± 0.12***	27.6	± 4.5***
13	TB	17.4	± 2.4**	0.79	± 0.11***	30.2	± 4.2***
14	BHT/CC	240.8	± 27.1***	11.28	± 1.56***	502.7	± 74.4***

15	BHT/PG	378.9	± 48.6***	17.13	± 2.25****	747.0	± 98.6***
16	BHT/TB	325.2	± 65.3***	15.45	± 3.07***	662.5	± 108.0***
17	CC/PG	26.0	± 5.3***	0.97	± 0.20***	37.4	± 7.8***
18	CC/TB	19.1	± 4.2***	0.88	± 0.21***	34.6	± 8.3***
19	PG/TB	12.4	± 1.3	0.49	± 0.04	19.3	± 1.5*
20	BHT/CC/PG/TB	86.6	± 8.2***	3.76	± 0.48***	159.2	± 22.4***
21	BHT/CC	105.0	± 13.6***	4.22	± 0.62***	165.9	± 24.1***
22	BHT/PG	97.3	± 8.7***	3.99	± 0.31***	158.9	± 11.0***
23	BHT/TB	67.5	± 5.3***	2.85	± 0.28***	118.9	± 10.3***
24	CC/PG	13.2	± 1.4	0.52	± 0.04*	19.9	± 1.5*
25	CC/TB	12.4	± 1.7	0.53	± 0.07*	21.0	± 2.8**
26	PG/TB	11.2	± 0.7	0.45	± 0.02	17.5	± 1.1
27	BHT/CC/PG/TB	25.4	± 3.4***	1.01	± 0.12***	41.0	± 5.2***
28	BHT/CC/PG/TB	13.7	± 1.0*	0.50	± 0.04*	20.0	± 1.6*

[a]For details of the dietary levels of BHT, CC, PG and TB see Table 18.
[b]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats.
Values significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

The effect of treatment of rats for 28 days with various dietary combinations of BHT, CC, PG and TB on various dietary levels of CYP1A2 mRNA is shown in Table 25. For the single compound exposures the most marked increases were observed with the three dose levels of TB studied. While treatment with BHT had no significant effect on hepatic CYP1A2 mRNA levels, small increases were observed in rats given 4062 ppm CC and 762-3046 ppm PG. Levels of CYP1A2 mRNA were increased in rats treated with some of the binary and quaternary mixtures of the food additives (Table 25). In two instances (Groups 24 and 28) levels of CYP1A2 mRNA were somewhat lower than in the control animals.

The treatment of rats with 762-3046 ppm BHT produced marked dose-dependent increases in CYP2B1 mRNA levels (Table 26). Levels of CYP2B1 mRNA were also increased in rats given 1750 ppm TB and 1016 ppm CC. Significant increases in CYP2B1 mRNA levels were also observed in rats treated with the majority of the binary mixtures of the food additives and with all three quaternary mixtures studied (Table 26).

Liver cytosolic fractions from all 174 rats were assayed for protein content (Table 27) and GST activity towards CDNB (Table 28) and DCNB (Table 29) as substrates. GST

activity towards CDNB and DCNB as substrates was expressed as specific activity (i.e. per unit of cytosolic protein), per gram of liver and per liver weight per kg body weight. Treatment with the single compound exposures and all the binary and quaternary mixtures studied had no statistically significant effect on hepatic cytosolic protein content (Table 27).

GST activity towards CDNB as substrate (Table 28) was significantly increased in rats given 1523 and 3046 ppm BHT (Groups 3 and 4) and 875 and 1750 ppm TB (Groups 12 and 13). These increases were significant when GST activity was expressed per unit of cytosolic protein, per gram of liver and per liver weight per kg body weight. The greatest induction of GST activity was observed with 3046 ppm BHT (Group 4) where enzyme activity expressed as specific activity, per gram of liver and per liver weight per kg body weight was increased to 262, 244 and 322% of control, respectively (Table 28). Significant increases in GST activity towards CDNB as substrate were also observed after treatment with many of the binary and quaternary mixtures of the food additives. These included most of the mixtures containing BHT and/or TB (Table 28).

As shown in Table 29 below, treatment with all three single compound exposure levels of BHT and TB produced statistically significant dose-dependent increases in GST activity towards DCNB as substrate. The greatest induction of enzyme activity occurred in rats given 3046 ppm BHT. In contrast to the effects of BHT and TB, treatment with the three single compound exposure levels of CC and PG had no significant effect on GST activity towards DCNB as substrate (Table 29). However, statistically significant increases in GST activity were observed with the majority of the binary mixtures of the food additives. In addition, when enzyme activity was expressed as either specific activity or per liver weight per kg body weight, statistically significant increases in GST activity were also observed with the three quaternary mixtures of the food additives (Table 29).

Some preliminary studies were performed to try to identify GST form mRNA levels that could be used as biomarkers of effect of the food additives in the *in vivo* mixtures study. Levels of GSTM2 and GSTP1 mRNA were determined in liver samples from control rats and from rats given 762-3046 ppm BHT, 1016-4062 ppm CC, 762-3046 ppm PG and

438-1750 ppm TB in the diet for 28 days. These two GST forms were selected on the basis of the results of the *in vivo* study with the individual food additives, where five dose levels of each test compound were examined. The results of the transcriptomics analysis (see Section 6.2.) suggested that BHT induced a GST form described as the Yb2 subunit. Based on the current nomenclature, this rat GST form is designated as GSTM2 (Eaton and Bammler, 1999). In addition, the *in vivo* study with the individual food additives demonstrated that high dose levels of BHT and TB induced GSTP1 mRNA levels (see Section 6.1.). As for the CYP form mRNA levels determined (see above), levels of GST form mRNAs were expressed as a ratio to albumin mRNA, which was co-amplified in a duplex reaction.

The treatment of rats with the three single compound exposures of BHT and PG had no statistically significant effect on levels of either GSTM2 or GSTP1 mRNA (Table 30a).

Table 29. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on hepatic GST activity towards 1,2-dichloro-4-nitrobenzene (DCNB) as substrate

Group	Treatment[a]	GST activity[b]								
		(nmol/min/ mg protein)			(µmol/min/g liver)			(µmol/min/liver kg body weight)		weight/
1	Control	87	± 2.7		9.0	± 0.33		335	± 10.5	
2	BHT	114	± 4.2	*	11.3	± 0.66	*	464	± 25.6	**
3	BHT	145	± 8.3	***	15.1	± 1.28	***	651	± 65.1	***
4	BHT	204	± 15.3	***	19.2	± 1.22	***	939	± 58.3	***
5	CC	93	± 3.8		9.4	± 0.59		352	± 20.6	
6	CC	88	± 7.2		9.6	± 0.68		367	± 24.1	
7	CC	83	± 8.6		8.8	± 0.67		335	± 28.3	
8	PG	91	± 6.6		9.3	± 0.50		344	± 14.8	
9	PG	106	± 6.0		10.7	± 0.58		398	± 22.8	
10	PG	104	± 4.8		10.3	± 0.63		357	± 18.2	
11	TB	117	± 8.4	**	11.3	± 0.99	*	451	± 28.2	**
12	TB	135	± 6.3	***	14.0	± 0.84	***	530	± 16.8	***
13	TB	157	± 10.4	***	16.3	± 1.01	***	622	± 37.1	***
14	BHT/CC	165	± 9.8	***	15.5	± 0.66	***	690	± 38.5	***
15	BHT/PG	156	± 8.6	***	14.7	± 0.83	***	641	± 36.6	***
16	BHT/TB	165	± 12.4	***	16.1	± 1.57	***	706	± 52.2	***
17	CC/PG	107	± 5.9		10.4	± 0.29		400	± 19.0	
18	CC/TB	132	± 11.0	***	13.5	± 1.47	***	530	± 51.0	***

19	PG/TB	133	± 5.9	***	13.1	± 0.74	***	520	± 25.9	***
20	BHT/CC/PG/TB	157	± 7.4	***	15.4	± 1.31	***	647	± 53.6	***
21	BHT/CC	138	± 7.7	***	13.3	± 1.09	***	520	± 37.4	***
22	BHT/PG	142	± 10.9	***	13.3	± 0.56	***	534	± 27.1	***
23	BHT/TB	158	± 8.0	***	15.2	± 1.22	***	640	± 55.3	***
24	CC/PG	110	± 9.0	*	10.5	± 0.35		403	± 14.3	
25	CC/TB	154	± 14.1	***	15.0	± 0.77	***	606	± 32.8	***
26	PG/TB	122	± 6.8	***	11.6	± 0.94	*	450	± 29.1	**
27	BHT/CC/PG/TB	128	± 6.9	***	12.2	± 0.75	**	492	± 23.1	***
28	BHT/CC/PG/TB	114	± 8.8	*	11.1	± 0.84		446	± 37.7	*

[a]For details of the dietary levels of BHT, CC, PG and TB see Table 27.
[b]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats.
Values significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

In contrast, treatment with the highest single compound exposure level of TB resulted in a significant increase in GSTM2 mRNA levels. Treatment with the lowest single compound exposure level of CC also resulted in a small increase in GSTP1 mRNA levels. Because of the lack of effect of the individual food additives on the two GST mRNA levels selected (Table 30a), no studies were performed with liver samples from rats given either binary or quaternary mixtures of the food additives.

6.4. Transcriptomics Analysis of the Hepatic Effects of Mixtures of Food Additives in the Rat

Gene expression analysis was performed using Affymetrix GeneChip Rat Expression 230A arrays. After quality control, normalisation and filtering, the data obtained from 48 microarrays involving the measurements of 10906 filtered probe sets were included in subsequent analysis. Fig. 2 displays the data analysis strategy. First, global gene expression patterns were considered, making use of this total dataset. In Fig. 8 results of hierarchical clustering analysis are shown. In Fig. 9, results of principal component analysis are shown. Based on these results of global expression analysis the following conclusions can be drawn:

- The effect of particular treatment appears to overrule the dose effect (genes are grouped together based on specific treatments rather than on treatment doses), at least

within the dosage regimen tested here. However, this conclusion is concerning the global gene expression pattern, and for individual genes dose dependencies may still exist.

- Gene expression profiles obtained from binary mixtures containing TB cluster together (Fig. 8). Also, note that in Fig. 9 the light blue dot representing TB at 100 is most close to the dot representing binary mixtures containing TB. This suggests that TB has most profound effects on global gene expression changes within these binary mixtures, at least within the dosage regimen tested here
- Treatment with curcumin (CC) has the weakest effect on transcriptome changes compared to other treatments, since the expression profiles obtained after CC treatment based on PCA appeared to be most similar to the control set: the light purple and dark blue dot representing treatment CC_100 and treatment CC_50 are most close to the red dot represent control (Fig. 9). Also, note in Fig. 8 that gene expression profiles after CC treatment cluster most closely to control.
- Even though CC and PG as individual additives only affected gene expression changes in a modest way, the binary mixture of CC and PG at fractional dosages of 50% each (CC_PG100), displayed a gene expression profiling quite distinct from control (light orange dot 'treatment CC_PG100'). This may suggest the occurrence of gene expression changes specific for this mixture that can not be explained by the effects of individual components on gene expression.

Next part of the data analysis involved the analysis of number of differentially expressed genes between treatment groups and control group. This was done by means of Cyber-T software (Baldi and Long, 2001). Cyber-T algorithm employs statistical analyses based on regularized t-tests that use a Bayesian estimate of the variance among gene measurements within an experiment. A total number of 3538 genes were considered of being differentially expressed in any of the treatments based on application of the Cyber-T algorithm. In Fig. 10 number of differentially expressed genes for each of the treatments after applying such significance threshold is shown. Although no statistical modelling was performed for testing of deviation from effect addition or dose addition using the gene expression values for single genes as starting point, - as was the case for conventional endpoints- analysis of the total number of genes that are differentially

expressed upon treatment with BHT, CC, PG, TB and their mixtures suggests the following:

- Concerning the number of differentially expressed genes, treatment with mixtures appears to have a stronger effect on hepatic gene expression than the treatment with the single compounds comprising these mixtures, primarily whenever comparisons to BHT, CC and PG are concerned. This holds true upon comparison of binary mixtures with total fractional dosage of 100 (e.g. CC at 50% together with PG at 50% (CC_PG100) results in a higher number of differentially expressed genes in comparison to the sum of differentially expressed genes caused by CC and PG at 50% or 100% fractional dosage alone). This suggests that for some binary mixtures, the effects of mixture exposure on gene expression changes, here defined as number of differentially expressed genes, can not be simply explaining by the mere increase in fractional compound levels as such.
- Treatment with thiabendazole (TB) causes the largest transcriptome changes compared to individual treatments with BHT, CC or PG, with number of differentially expressed genes higher or approximately equivalent than the number observed in binary and quaternary mixtures. This is in line with the observations derived from PCA and cluster analysis of global gene expression profiles, from which it was concluded that TB had the most profound effects, also in binary mixtures. Whenever binary mixtures are considered in more detail, using the number of differentially expressed genes as parameter, addition of either BHT, CC, PG at 50 % fractional dosage each to TB at fractional dosage of 50%, results in a reduction of the number of differentially expressed genes invoked by TB at fractional dose levels of both 50% and 100%. This suggests that addition of the other additives may suppress the effects induced by TB on the number of differentially expressed genes.
- Doubling of the BHT dose results in doubling of number of affected genes. This dose effect, here defined as the total number of differentially expressed genes, is not so observed upon treatment with CC, PG and TB

To further understand the correlation/distinction in gene expression profiles between the different treatments, genes that were differentially expressed in any of the treatments based on application of the Cyber-T algorithm (3538 genes in total) were used to

calculate correlation coefficients in all pair wise combinations. The results are represented as a correlation heatmap shown in Fig. 11.

The highest correlations (indicated by blue frames in Fig. 11) were observed within following treatment groups:

1. BHT group, comprising of BHT_50, BHT_100 and CC and PG binary mixtures containing BHT but not TB.
2. Binary mixtures, containing TB and the binary mixture of CC_PG at 50% fractional dosage for each of the compounds)
3. Q_TB_PG group, comprising Quaternary mixtures and TB and PG as individual compounds in both doses.

The lowest correlations (green frames in Fig. 11) were observed between:

- treatments in groups 1 to 3 and the CC_50 treatment
- treatments in group 2 and group 3 (with exception of TB treatment)

Based on results of the correlation analysis, taken the number of differentially expressed genes between any of the treatment groups and the control group as a starting point, treatment effects for each compound can be summarized as follows:

- CC - no effect, similar to control (the actual lack of correlation between CC and control, which could follow from this Figure, can be explained here by the fact that the most differentially expressed genes between CC and control, from Cyber-T analysis, were employed in the correlation analysis).
- BHT – has more pronounced effects, in comparison to CC, and is similar to BHT_CC100 mixture
- BHT - different effect than the TB-containing binary mixtures
- PG - similar effect as the quaternary mixtures (and to some extent TB)
- PG - different effect than the binary mixtures
- TB - similar effect to quaternary mixtures, dominant in binary mixtures

The results of correlation analysis suggest that the effect of TB is dominant to the effects observed upon treatment with binary mixtures, and that this effect differs from effect of BHT. These results are in line with the conclusions from cluster analysis and PCA. The

effect of binary mixtures is different from the effect of quaternary mixtures. This may be the consequence of the dose effect since the concentrations of single compounds are lower in quaternary than in binary mixtures.

The third part of the data analysis is concerned with quantitative analysis of treatment effects by determining the overlap in number of differentially expressed genes between the conditions tested. This analysis is concerned with further studying mixture effects, as well as understanding the contribution of individual components to the mixture effect. To further investigate if mixture effects are likely to occur and if mixture effects are primarily due to one compound, lists of differentially expressed genes obtained from Cyber-T analysis were compared with one another. Venn Diagrams were created to look for overlap and dissimilarity in gene expression, with emphasis on genes exclusively expressed in the mixtures only, the sum of genes expressed in each of the individual compounds only and genes expressed in both the mixture and any of the individual compounds. The number of differentially expressed genes specific to the mixture, specific to individual components comprising the mixture, and common to both mixture and individual components comprising the mixture are shown in Fig. 12. Comparison of these values illustrates how far mixture effects on gene expression can be explained from the individual components only, or from the combined effects of its individual components. The results show that mixture-specific effects, here defined as differentially expressed genes that occur in the mixtures only, can be expected to occur for each combination of investigated compounds (light yellow and red parts of the bars).

A particular effect is suggested in CC_PG_100 compared to treatment with either CC or PG separately. Notice that while the number of differentially expressed genes by CC and PG as single compounds at fractional dose levels of 50 and 100, is only modest, exposure to the binary mixture of CC at 50 and PG at 50 (CC_PG100) results in a much larger number of differentially expressed genes, than would be expected on the summation of the number of genes differentially expressed by CC and PG as individual compounds. Therefore, it appears that a clear mixture effect may be observed for CC_PG_100, not observed upon exposure to the individual compounds, both at fractional dose levels of 50% and 100%. Further, it is not likely that this is due to the simple increase in compound load as such, since comparison of CC_PG_100 with both individual

compounds at fractional dose levels of 50% and 100% leads to the same conclusion (yellow and red bars). The number of differentially expressed genes observed with this mixture is almost 3 fold higher in comparison to the sum of effects of its single compounds.

In contrast, binary mixtures containing TB have a relatively small effect that is specific to the mixture only, compared to the effect of treatment with single components, likely due to the dominant effect of TB.

To investigate which compounds in the mixture contributes most to the mixture effects, number of overlapping differentially expressed genes between the mixture and each of the mixture components were compared (Fig. 13). Thus, the analysis below focuses on genes that are differentially expressed in the mixtures, as well as any of the individual components, and determines the contribution of each of the single compounds to the number of differentially expressed genes observed upon mixture exposure. The analysis does not include genes that are exclusively differentially expressed in the mixture only, discussed in Fig. 12. The results in Fig. 13 reveal the following, considering genes that were found to be differentially expressed in the mixtures jointly with differential expression in any of the individual compounds comprising the mixture:

- The effect of TB on hepatic gene expression is dominant to the effects of other compounds in TB-containing mixtures,
- Gene expression changes indicating this predominant effect of TB in mixtures likely can not be explained by the simple presence of more compound load as such within the dose range tested, since comparison of binary mixtures at total fractional dose levels of 100 (here for example indicated as CC_TB100) with single compounds at both 50 and 100 at fractional dose levels, respectively still yields the same conclusions.
- The effect of BHT is dominant to the effects of CC and PG in BHT_CC100 and BHT_PG100 mixtures. This also follows from correlation analysis in Fig. 11.
- CC has small impact within CC_TB100 and CC_BHT100 mixtures
- CC and PG contribute equally to the CC_PG100 mixture effect, in terms of number of differentially expressed genes, and on top of this displayed the relative largest number of novel mixture specific gene expression changes. This could be indicative

for the activation of interactive mechanisms. So, in a binary mixture of CC and PG, with both compounds at fractional dosage of 50% each (CC_PG100), differential gene expression can be partially explained by a set of genes of which half is already differentially expressed upon CC treatments, and half is already differentially expressed upon PG treatments. Next to this, CC and PG showed the relative largest number of differentially expressed genes that are exclusive to this binary mixture (CC_PG100) only.

Finally a limited, functional analysis is performed on microarray data. This is additional to the Scope of Work submitted to FSA in April 2006 and involves limited activities towards biological analysis of gene expression data in single compounds and mixtures. To discover biological processes that are affected by treatment with BHT, CC, PG, TB and their mixtures, functional analysis of gene expression changes was performed using T-profiler (Boorsma et al., 2005). This tool enables calculation of significance (t-value) of the relative upregulation (positive t-values) or downregulation (negative t-values) of genes, compared to control, within a predefined group that either refers to a biological processes (P), molecular function (F) or cellular component (C); groups of genes classified by the gene ontology (GO) consortium (www.geneontology.org), in comparison to the total gene expression set. This analysis provides information which biological processes are changed upon exposure to additives and mixtures thereof. Table 30b below displays the statistically significant Gene Ontology categories (with E-value <0.05).

Table 30b. **T-profiler analysis showing enriched gene ontology categories in single compound and mixture exposures.**

Treatment		t-value	E-value	mean	Orfs
BHT_50	glutathione transferase activity - F	4.52	0.0067	0.3	13
BHT_100	glutathione transferase activity - F	6.45	0	0.52	13
	metabolic process - P	5.6	0	0.11	205
	xenobiotic metabolic process - P	4.54	0.0062	0.46	8
PG_50	immune response - P	-4.5	0.0074	-0.15	69
TB_50	unfolded protein binding - F	4.31	0.0178	0.31	34
TB_100	microsome - C	4.47	0.0087	0.23	79
	unfolded protein binding - F	4.28	0.0204	0.31	34
	electron transport - P	4.09	0.0456	0.17	149
	complement activation, classical pathway - P	-4.14	0.0373	-0.41	12
	innate immune response - P	-4.56	0.0056	-0.43	13
	immune response - P	-5.16	0.0003	-0.19	69
	phosphate transport - P	-5.2	0.0002	-0.33	27
BHT_CC_100	glucuronosyltransferase activity - F	4.15	0.0356	0.69	4
BHT_TB_100	oxidoreductase activity - F	6.17	0	0.83	7
	microsome - C	4.96	0.0008	0.21	79
	glucuronosyltransferase activity - F	4.91	0.001	0.88	4
	glutathione transferase activity - F	4.41	0.0116	0.44	13
CC_PG_100	transcription - P	4.82	0.0016	0.09	194
	regulation of transcription, DNA-dependent - P	4.67	0.0032	0.06	340
	sequence-specific DNA binding - F	4.26	0.0228	0.12	101
CC_TB_100	fatty acid metabolic process - P	4.88	0.0012	0.29	37
	response to unfolded protein - P	4.45	0.0092	0.49	11
	sequence-specific DNA binding - F	4.26	0.0221	0.15	101
All_100	translation - P	4.14	0.0374	0.14	98

For example the BHT_100 group displays a highly significant t-value for the category glutathione transferase activity, indicating that compared to untreated control animals, the majority of genes in this class are upregulated, in comparison to the total number of genes on the array. Interestingly, the binary mixture CC_PG_100, for which comparison of differentially expressed genes suggested that specific mixture effects may occur, the gene ontology categories ‘transcription’, ‘regulation of transcription’, ‘sequence-specific DNA binding’ were significantly upregulated. This may suggest that, while these GO categories were not differentially expressed at fractional dosages of 50% and 100% of each of the individual additives, additional transcriptional mechanisms may be activated in this binary mixture leading to an increase in expression of novel genes. To further discover biological effects that are potentially enhanced or attenuated in mixtures compared to single components, t-values of selected GO categories from Table 30b where compared across all treatments. The

examples are shown in Fig. 14 below. Functional categories “Xenobiotic metabolic process” and “Glutathione transferase activity” are both upregulated upon treatment with BHT and these effects seem to be attenuated in the mixtures. On contrary, CC and PG have stronger effect on these two categories in CC_TB100 and PG_TB100 mixtures than as single components in either low or high doses. The significance profile of CC_TB100 and PG_TB100 is comparable to TB_100 values, suggesting that occurring mixture effects resemble the effect of TB enhancement. Functional category “Fatty acid metabolic process” is upregulated upon treatment with high doses of CC (non significant), high doses of TB or with CC containing binary mixtures (with the exception of BHT_CC100), whereas it is downregulated upon PG treatment.

Figure 14. T-profiler analysis on hepatic gene expression profiles obtained from rats exposed to individual compounds and mixtures of BHT, CC, PG and TB.

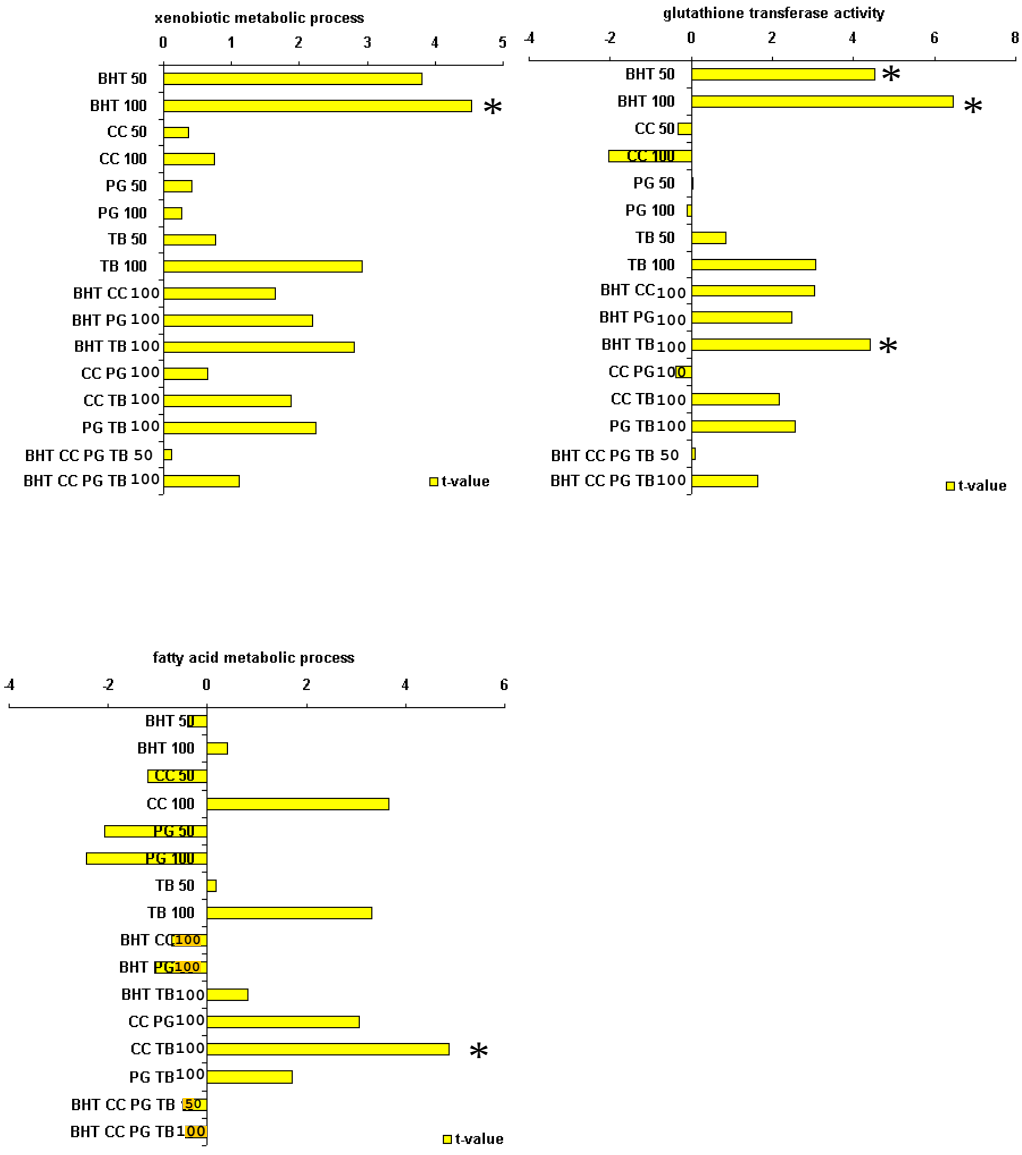

Fig. 14. Examples of gene ontology (GO) functional categories differently affected in mixtures compared to single components. Bars marked with an asterisk demarcate treatment(s) for which given GO category has statistically significant t-value.

6.5. Statistical Analysis of the Hepatic Effects of Mixtures of Food Additives in the Rat and Inference of Mixtures Effects

The differences between actual dosages, as determined by HPLC and nominal dosages were for all groups a maximum of 5 -10% (Table 15b), indicating that the experimental dose levels as determined in the diet (actual dosage) were close to the intended dose levels. A total of 174 animals were included in the study analysis. Food intake (g/rat/day) was, except for the highest dose group of TB, +/- 10% of control group. Thus, with the

exception of rats fed the highest dietary level of TB (target concentration 1750 ppm, actual concentration 1842 ppm), where a reduction in food consumption was noted, food consumption of all the experimental groups was generally similar to that of the controls. For selected animals (see 'Supplementary statistics.doc', supplementary Table S-1), values for parameters indicated were not included in the analysis, since these appeared to be outliers. All individual animal data are available in the supplementary electronic file 'DF1_Data Summary.xls'. The outcome of statistical analyses, that is the result of Student t-tests inferring for differences between measured values and predicted values based upon the Effect Additivity Surfaces Equations, for each parameter, is provided in the supplementary data files DF2-DF6. A detailed description of the statistical analysis, rationale and outcome, with reference to these supplementary data files, is provided in the supplementary document 'Supplementary statistics.doc', containing supplementary Tables 'S-1 through S-16'. Group averages for all parameters, including average $\pm$ SD, which is the observed averages, geometrical mean and predicted values, based upon the effect additivity surface equations derived from the dose response curves obtained from single compound exposures, are shown in supplementary Tables S-10 through S-16. The measured values (averages) were reported before in Tables 18-29.

Given the large amount of data and analyses, the overall outcome from the document 'Supplementary statistics.doc' are compiled in a comprehensive manner in
Fig. 15 through Fig. 19. Figures are shown for body weight (Fig. 15) and liver weight (Fig. 16), as well as for markers for Phase I and Phase II metabolism, for which significant interactions appeared to be present, which included GST activity towards CDNB (Fig. 17) and DCNB (Fig. 18) and CYP1A2 mRNA expression (Fig. 19). Outcome on other parameters for which no interactions were present (relative liver weight, total CYP content, 7PR, 7ER and CYP2B1 mRNA expression) are not condensed into Figures, but described in 'Supplementary statistics.doc' and reported in supplementary Tables S-10 through S-14, and shortly at the end of the results section. Next to the mixtures effects, the observed values for the single compound exposures, together with significant if any, relative to control are visualized. In Fig. 15 through Fig. 19, the relative value for each group is shown in direct comparison to control, both for the predicted (solid bars) and measured (grey bars) values. Predicted values for mixtures were based upon the additivity surface equations. First, under the header indicated by [A]

'*Effect addition Non additivity'*, the statistically significant deviations from effect addition for each of the mixtures, as inferred from Student t-test based comparisons of the measured values with predicted values from effect additivity surface models (see also paragraph 6 of the 'Supplementary statistics.doc' and raw data file 'DF3_additivitysurf.out'), are shown. These statistical comparisons are reported in raw data file 'DF4_nonadditivity.out' and discussed in further detail in paragraph 7 of 'Supplementary statistics.doc'. Under the header indicated by [B] '*Effect addition interactions'*, the outcome of a more detailed analysis characterizing any interactions, in terms of dose dependency is shown. This is derived from raw data file 'DF6_interaction.out' and described in further detail in paragraph 8 and Tables S-6 and S-7 in the 'Supplementary statistics.doc'. Under the header indicated by [C] *'Departure from dose addition'* the outcome for compatibility of the data with dose addition is shown, obtained from analysis described in supplementary Tables S-8 and S-9 contained within paragraph 9 in the supplementary document 'Supplementary statistics.doc'. Finally, under the header indicated by [D] *'Interaction'* overall conclusions are provided, taking into account the dose dependency of the individual additives as well as the overall test on departure from effect addition (the latter not shown in Fig. 15-Fig. 19, but reported in the far right column of supplementary Table S-5 in the 'Supplementary statistics.doc').

With respect to body weight (Fig. 15), despite the significant difference between the highest dose level of TB and control, no significant dose dependent effects were observed for each of the individual compounds (Also supplementary Table S-5, columns BHT, CC, PG and TB respectively and Table-S10). The effect additivity surface used to calculate the predicted body weight (in gram) was equivalent to: $\ln(BW)=5.699-0.197*[BHT]-0.0156*[CC]-0.0446*[PG]+0.0181*[TB]+ 0.1779*[BHT]^2-0.1718[TB]^2$. The overall test on deviation from effect addition was significant ($P<0.001$) (right column supplementary Table S-5), indicating that mixture groups exist for which the effect in the combined exposure was significantly different in comparison to the effects predicted from the individual exposures, from which the additivity surface equation was established. In more detail, minor deviations from effect addition were found for the BHT_TB50 group, with predicted values being 96% of control and measured values 104% of control. However, such an effect was absent in the BHT_TB100 group. For the

Figure 15. Outcome of statistical analysis for inference of mixture effects in rats exposed to individual compounds and mixtures of BHT, CC, PG and TB: body weight

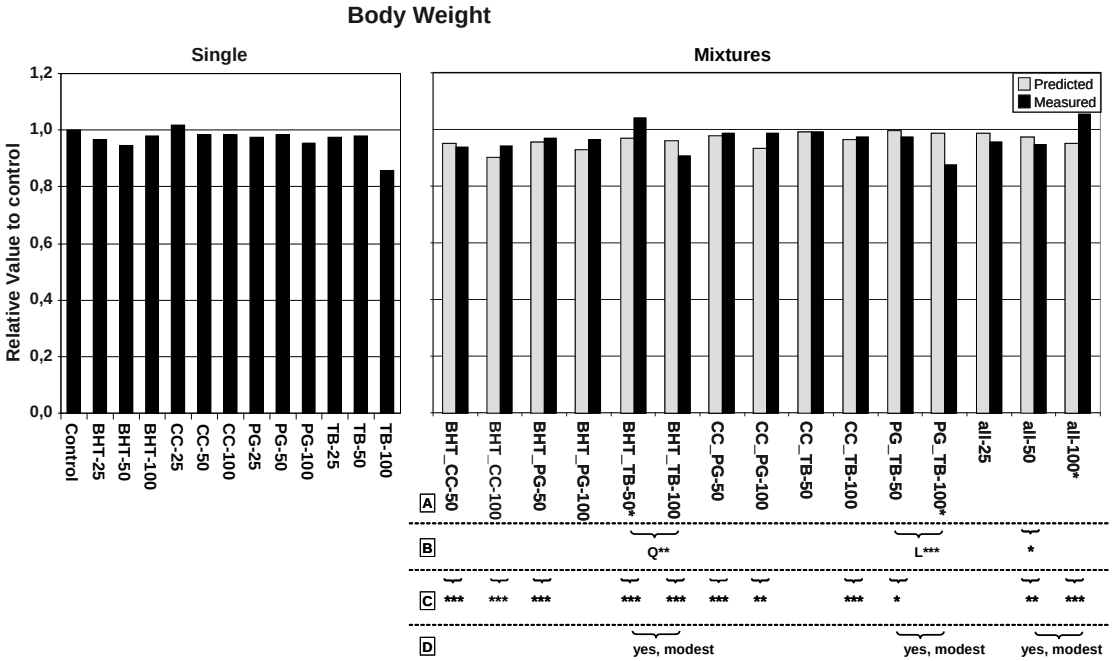

Fig. 15. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on body weight (expressed in g) in Sprague Dawley rats exposed for 28 days. Results are shown relatively to the observations in the control group. Left side represents the measured group averages in single compound exposures, relative to control values: 1: linear term contributing to dose dependency of individual additive; 2: quadratic terms contributing to dose dependency (see also supplementary Table S-5) of individual additive. Right side represents predicted and measured group averages for mixture exposures. Additivity Surface Equation used to calculate the predicted values for mixtures: $\ln(BW)=5.699-0.197*[BHT]-0.0156*[CC]-0.0446*[PG]+0.0181*[TB]+0.1779*[BHT]^2-0.1718[TB]^2$. Header [A] represents '*Effect addition Non additivity'*, the statistically significant deviations from effect addition for each of the mixtures. Header [B] represents '*Effect addition interactions'*, characterizing interactions in terms of dose dependency. Header [C] represents *'Departure from dose addition'* the outcome for compatibility of the data with dose addition. Header [D] represents *'Interaction'* overall conclusions. L: linear dose dependency of interaction; Q: quadratic dose dependency of interaction; o: $P < 0.10$; *: $P < 0.05$; **: $P < 0.01$; *** $P < 0.001$ (Student t-test).

PG_TB100 group, predicted value was 99% of control, whereas the measured value was 87% of control. Further characterization of these binary mixtures pointed towards statistically significant but modest interactions for these mixtures, since dose-dependency for the interaction between BHT and TB ('Q') was observed, meaning that the strength of the interaction increased with increasing dose of the individual compounds. The interaction for the mixtures of PG and TB, was non dose-dependent ('L'), (see also Supplementary Table S-6). Also, for both BHT_TB and PG_TB, departures from dose addition were observed, suggesting that interactions are indeed likely for these two binary mixtures. However, taking into account the magnitudes of these interactions, combinations of binary mixtures did not influence body weight to a great extent for all mixtures assessed. For all of the other binary mixtures, for most dose levels, departure from dose addition was observed as well, however, no significant deviation from the concept of effect addition was found. With respect to the quaternary mixtures, a modest interaction was observed and the all 100 group displayed slightly higher (~11%) body weights than expected body weight, and statistics indicated an interaction in the all-50 group as well. Thus, with respect to body weight, no major interactions are evident, expect for some modest interactions in binary mixtures of BHT and TB, PG and TB and in the all-100 quaternary mixture, respectively. For the majority of these additives and combinations thereof, the response of mixtures can be predicted from the simple addition of the effects invoked by the individual additives.

Regarding liver weight (Fig. 16), significant dose dependent effects were observed on liver weight with BHT and TB, as individual compounds, but not for the other compounds (also supplementary Table S-5, columns BHT, CC, PG and TB respectively and Table S-10). The effect additivity surface used to calculate the predicted liver weight (in gram) was equivalent to: $\ln(LW)= 2.4088+0.2449[BHT]+0.0122[CC] -0.0463[PG]+ 0.218[TB]-0.349[TB]^2$. The overall test on deviation from additivity was non significant (far right column Table S-5). Also, no departure from effect addition was found for any of the binary mixtures, but only for the all 100 quaternary mixture (under header [A]). Nevertheless, statistical modeling proposed a dose-dependent ('Q') interaction for the combination of BHT and CC, however at borderline significance ($P<0.10$) and for the quaternary mixture of all compounds at a 100 dose level (under header [B]). For this treatment group, the predicted value was 108% of control, whereas

Figure 16. Outcome of statistical analysis for inference of mixture effects in rats exposed to individual compounds and mixtures of BHT, CC, PG and TB: liver weight

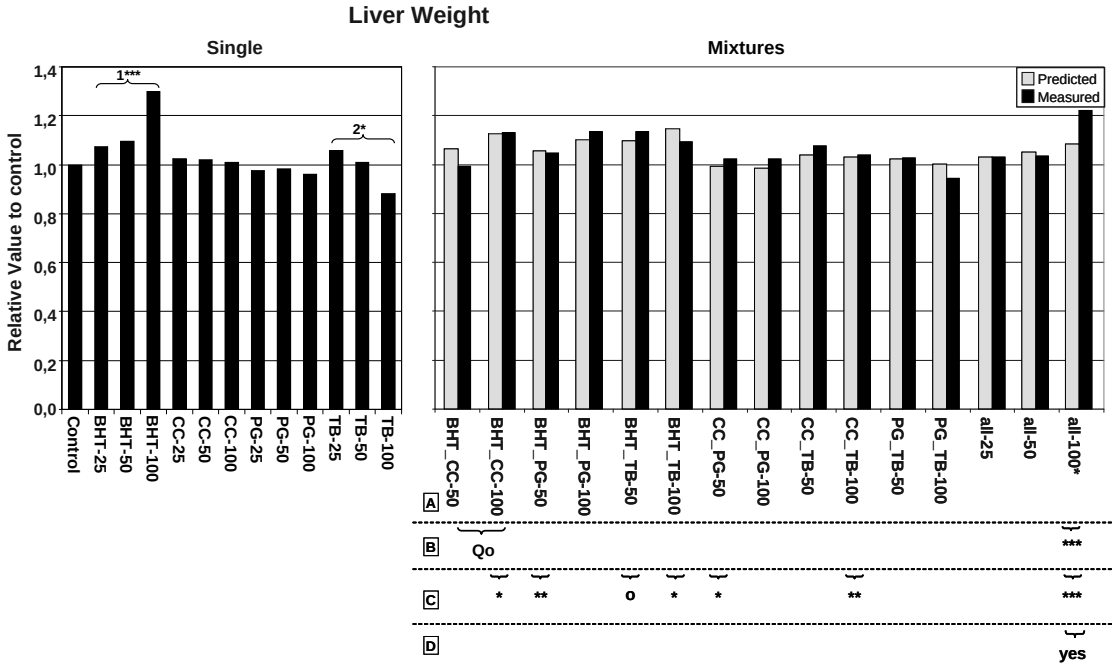

Fig. 16. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on liver weight (expressed in g) in Sprague Dawley rats exposed for 28 days. Explanation is similar as in the legend of Fig. 15. Additivity Surface Equation used to calculate the predicted values for mixtures: ln(LW)= 2.4088+0.2449[BHT]+0.0122[CC] -0.0463[PG]+ 0.218[TB]-0.349[TB]^2.

observed value for this quaternary mixture was 122 % of control. As with body weight, for most other binary mixtures, departure from dose addition was observed. Overall, based on the outcome of liver weight as parameter, except for the all 100 mixture group in which measured liver weights were higher than expected, interaction between mixtures with respect to this endpoint are not evident. Therefore, with respect to liver weight, the concept of simple addition for these additives seems more likely than the concept of dose addition. In terms of interactions, results expressed as relative liver weight were essentially identical as for liver weight (not shown in this report, but available in supplementary Tables S-5 and S-6 and S-10).

Results for GST activities towards CDNB expressed as μmol/min/mg protein relative to control, are shown in Fig. 17. The additivity surface equation used to calculate the predicted values for mixtures was equal to: ln(CDNB)=0.8128+0.9836[BHT]-0.0813[CC]+0.0699[PG]+0.7298[TB].

BHT and TB had significant linear dose dependent positive effects (supplementary Table S-5 and Table-S15). Concerning the mixtures, the overall test on deviation from effect addition was significant (P<0.01) (right column supplementary Table S-5). Indeed, when GST activities towards CDNB for binary mixtures were considered in greater detail (under header [A]), statistically significant departures from effect addition were observed for combinations of BHT_CC (P<0.05), BHT_PG (P<0.05) and BHT_TB (P<0.05), at least in one of the two dose groups employed for each of the binary combinations. Measured values for GST activities towards CDNB, in these binary mixtures were generally 20-30% higher than the predicted values. When these data were considered in greater detail, indeed significant interactions were found for binary mixtures of BHT_CC (linear, non-dose dependent, P<0.05), BHT_PG (linear, non-dose dependent, P<0.05) and BHT_TB (quadratic, dose dependent P<0.001) groups (under header [B]). Further, the concept of dose addition was not compatible for these binary mixtures, at least significant for one of the two dose groups (BHT_CC100, BHT_PG100 and BHT_TB50). Therefore, it is likely that in binary mixtures of additives, containing BHT, interactions do occur with respect to GST activities towards CDNB. Further, even in absence of dose response effects of CC and PG as single compounds, linear (non-dose dependent) interactions were observed for binary mixtures CC_PG (P<0.05) and CC_TB (P<0.10, at borderline

Figure 17**.** Outcome of statistical analysis for inference of mixture effects in rats exposed to individual compounds and mixtures of BHT, CC, PG and TB: CDNB

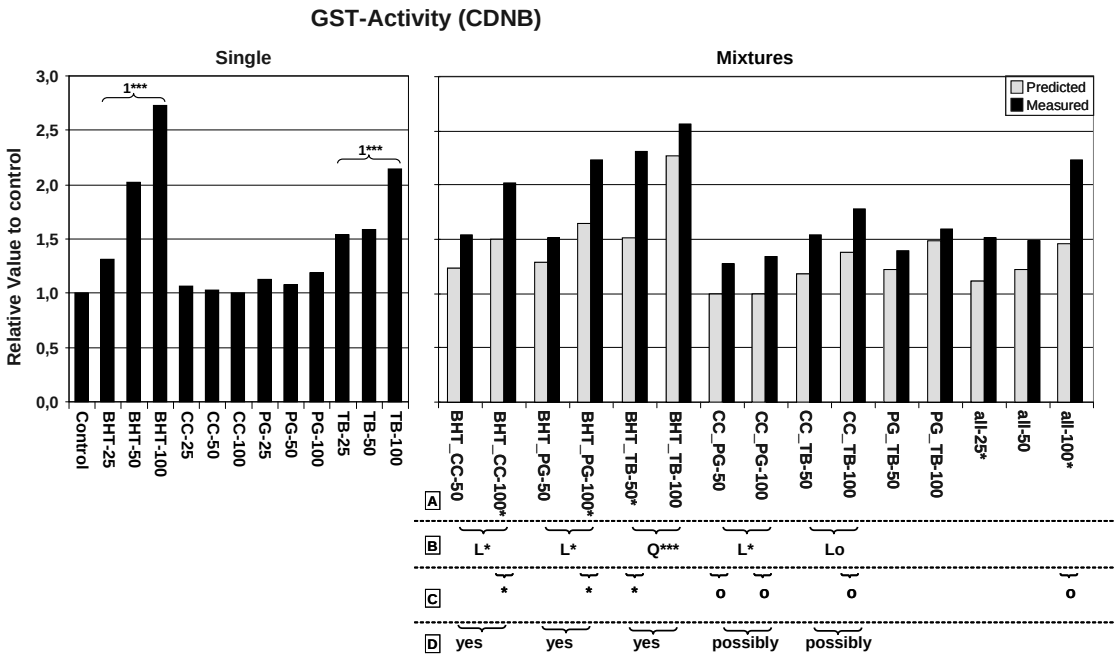

Fig. 17. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on GST activities towards CDNB (expressed as μmol/min/mg) in Sprague Dawley rats exposed for 28 days. Explanation is similar as in the legend of Fig. 15. Additivity Surface Equation used to calculate the predicted values for mixtures: ln(CDNB)=0.8128+0.9836[BHT]-0.0813[CC]+0.0699[PG]+0.7298[TB].

significance). For these two mixtures, a dose response model was only nearly compatible with the datasets (deviation from dose response model were borderline significant). Therefore, the presence of interactions on this parameter is not excluded. For the combination of PG and TB, no significant deviations from statistical models describing effect addition and dose addition were observed and no interactions are likely to occur for this parameter. The all 100 group also demonstrated deviation from effect addition, with predicted GST activities towards CDNB of 147% of control in comparison to a measured activity of 223% of control.

The results on hepatic glutathione S-transferase activity towards DCNB (expressed as nmol/min/mg) are shown in Fig. 18, relative to control (all group averages shown in Table S-16). The additivity surface equation used to calculate the predicted values for mixtures: ln(DNCB)=4.5068+0.8355[BHT]-0.1073[CC]+0.152[PG]+1.071[TB]-0.533[TB]^2. With respect to the effects of the single compounds, GST activities towards DCNB in animals were significantly associated in a linear (BHT and PG) and quadratic (TB) manner with the dose level employed (Supplementary Table S-5). Considering the mixtures, the overall test on deviation from effect addition was highly significant ($P<0.001$, right column Table S-5), indicating presence of mixture groups for which GST activities towards DCNB are not compatible with effect addition. Indeed, a more detailed assessment showed significant positive departure from effect addition for binary mixtures of BHT and CC, with a predicted and measured increase in GST activity towards DCNB compared to control of 118-139% and 158-188%, respectively (under header [A]) and a dose-dependent synergistic interaction ('Q*, at $P<0.05$') was observed for the combination of BHT and CC, within dose ranges employed (under header [B]). Also, the data were not compatible with the concept of dose addition, since significant departure from dose addition was observed (under header [C]). Therefore, even though CC by itself did not have a significant effect on GST activities towards DCNB (Supplementary Table S-5, under column CC), an interaction is likely with respect to GST activities towards DCNB for the combination of BHT and CC, in line with the interaction already predicted from the overall GST activity using CDNB as substrate. Similar findings were observed for the combinations of BHT and PG, CC and PG and CC and TB, respectively, with higher measured relative levels of GST activities towards DCNB, in comparison to predicted (for each of these binary mixtures, significant in at least one dose group). In

Figure 18. Outcome of statistical analysis for inference of mixture effects in rats exposed to individual compounds and mixtures of BHT, CC, PG and TB: DNCB

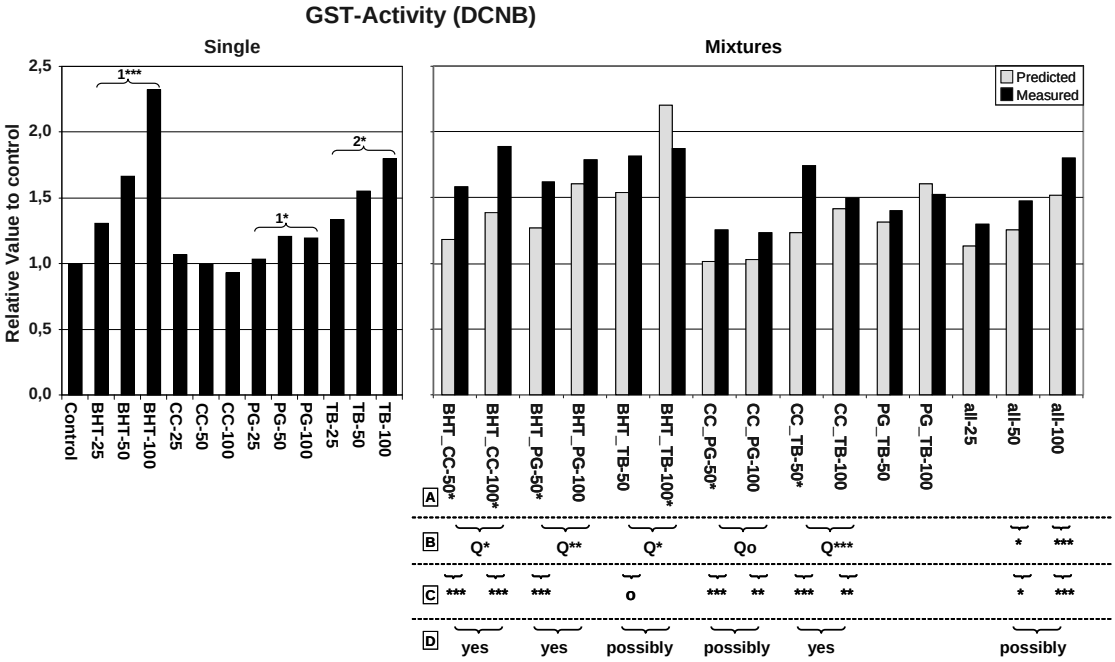

Fig. 18. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on GST activities towards DCNB (expressed as nmol/min/mg) in Sprague Dawley rats exposed for 28 days. Explanation is similar as in the legend of Fig. 15. Additivity Surface Equation used to calculate the predicted values for mixtures: $\ln(DNCB)=4.5068+0.8355[BHT]-0.1073[CC]+0.152[PG]+1.071[TB]-0.533[TB]^2$.

line, dose-dependent interactions ('Q') were proposed for these mixtures (header [B]). Further, for most of these groups, datasets were not in line with the concept of dose addition. Therefore, in mixtures of BHT and PG, CC and PG, and CC and TB, respectively, interactions occur, in which the effect of combined exposures on GST activities towards DCNB is increased in comparison to predicted values. For the binary mixture of BHT and TB, measured relative GST activities towards DCNB, in the 50 and 100 total dose groups, were slightly higher and lower respectively, compared to relative predicted levels and a significant dose dependent interaction ('Q*') was observed. Deviation from dose addition was at borderline significance in the BHT_TB50 group (under header [C]). Therefore, an interaction is not excluded for BHT and TB although the direction is less clear. With respect to the binary mixture of PG and TB, as for GST activities towards CDNB described above, both models (effect addition and dose addition) comply with the datasets, implying that no interactions are likely to occur. Further, for quaternary mixtures at the two highest total dose levels, interactions were likely, with a relative increase in GST activities towards DCNB, compared to predicted levels, however not exceeding the magnitude of interactions already observed in some of the binary mixtures. CYP1A2 mRNA expression was the parameter for which the most pronounced differences between predicted and measured values were found (Fig. 19, Table S-5, S-6, S-14). The Additivity Surface Equation used to calculate the predicted values for mixtures was equal to: $\ln(CYP1A2)=0.042+0.312[BHT]+0.551[CC]+4.16[PG]+8.59[TB]-2.92[PG]^2-6.02[TB]^2$. Concerning the individual additives, both PG and TB had a significant dose dependent effect on CYP1A2 expression (Table S-5). With regard to the mixtures, the overall test on deviation from effect addition was highly significant ($P<0.001$) (Supplementary Table S-5 far right column 'non-additivity). Indeed, significant deviations from effect addition were found for nearly all binary mixtures (under [A]), the majority of mRNA levels for these mixtures being significantly lower in comparison to predicted mRNA levels. It is interesting to notice that PG_TB groups, which as individual compounds had significant dose dependent positive effects on CYP1A2 (Supplementary Table S-5), also showed the relative largest induction of CYP1A2 compared to control, however, at much lower levels than would have been expected based upon individual compound exposure: negative departure from effect addition was strongest for this binary mixture. Moreover, for all of these binary and quaternary combinations, significant antagonistic interactions

Figure 19**.** Outcome of statistical analysis for inference of mixture effects in rats exposed to individual compounds and mixtures of BHT, CC, PG and TB: CYP1A2 mRNA

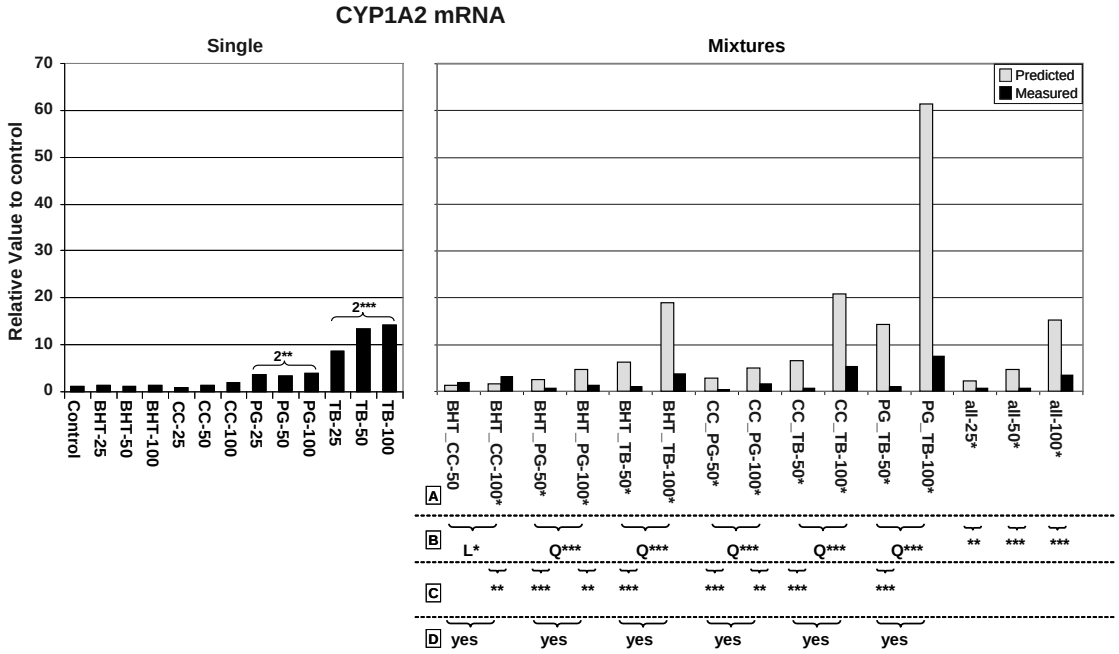

Fig. 19**.** Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on CYP1A2 mRNA levels (fold induction compared to control) in Sprague Dawley rats exposed for 28 days. Explanation is similar as in the legend of Fig. 15. Additivity Surface Equation used to calculate the predicted values for mixtures: $\ln(CYP1A2)=0.042+0.312[BHT]+0.551[CC]+4.16[PG]+8.59[TB]-2.92[PG]^2-6.02[TB]^2$.

were indeed observed which, except for the BHT + CC combinations, appeared to be dose dependent ('Q') (under [B]). Also, for all of these binary mixtures, at least one of the dose groups was not compatible with the concept of dose addition (under [C]). Therefore, it is likely that antagonistic interactions occur between these mixtures with respect to CYP1A2 mRNA expression.

With respect to other parameters, in general no or only modest (in terms of number of mixture groups or magnitude) interactions were observed. The details are presented in the supplementary materials, and shortly summarized below. No interactions between BHT, CC, PG and TB were evident for relative liver weight (Table S-5 and S-6); generally simple effect addition applied. Regarding total CYP content, dose dependency was observed upon BHT treatment (Table S-5). Measured CYP content for BHT_CC50 mixture was 90% of predicted CYP content (expressed as nmol/mg protein, dose dependent interaction) (Table S-6 and Table S-11) and departure from dose addition was observed (Table S-9). For the other binary mixtures, both models describing effect and dose addition were compatible with the observed data. Significant dose dependent effects were observed on 7PR for all additives (Table S-5, Table S-13). Given the problems with modelling of the dose response curve for CC as individual additive, related to the aberrant behavior of the CC25 dose group ('Supplementary statistics.doc', paragraph 6 and 7), no conclusions on binary and quaternary mixtures containing CC are provided. The overall test on deviation from effect additivity was highly significant ($P<0.001$) (Supplementary Table S-5 and Table S-6). Departure from effect addition was found for both PG_TB mixtures with measured activities being 57%-69% of predicted enzyme activities (derived from Table S-13). The interaction was linear (Table S-6), and not compatible with dose addition (Table S-9). Therefore, although modest, interaction for PG_TB mixtures on 7PR activity is likely. For BHT_PG and BHT_TB, data are more compatible with effect addition, as opposed to dose addition. Concerning 7ER (group average data in Table S-12), the overall test on deviation from effect addition for all mixture groups was non-significant (Table S-5), independently how 7ER was expressed. However, statistical analysis on plain activity data proposed a significant dose-dependent interaction ('Q*') for BHT_TB mixtures (Table S-6). However, at both dose levels of binary mixtures of BHT and TB, differences between predicted and observed values were small and no significant deviation from effect addition was observed (see data file:

DF4_nonadditivity and Table S-12). Further, the combination of these two additives was also compatible with the concept of dose addition, for both dose groups, making a true interaction between these additives with respect to this parameter unlikely. For all of the other mixtures, no significant deviations from effect or dose addition existed (Table S-6, S-8, S-9). All food additives demonstrated a dose dependent effect on CYP2B1 mRNA expression levels (Table S-5). Given the problems for CC25 and PG50 with respect to this parameter ('Supplementary statistics.doc', paragraph 6 and 7), description of results here is confined to mixtures containing BHT and TB only. No interactions were observed for BHT_TB mixtures (Table S-6). Departure from dose addition was significant in the BHT_TB100 group (Table S-8), whereas departure from effect addition for both dose groups was not, arguing that the effects of BHT and TB with respect to this parameter comply with simple effect addition.

6.6. Rat in vitro Studies with Individual Food Additives

Male Sprague-Dawley rat hepatocytes were cultured in a "sandwich" configuration and treated with BHT, CC, PG, TB and the reference compounds for 72 hours. The reference compounds consisted of known inducers of CYP forms and two known cytotoxic agents. Preliminary experiments were performed to assess the solubility of the four food additives in tissue culture medium and to identify suitable concentration ranges for subsequent studies. The overall concentration ranges examined were 0.5-200 μM for BHT and TB and 0.5-50 μM for CC and PG. Wider concentration ranges of BHT and TB were examined than for CC and PG as previous *in vivo* studies had demonstrated that BHT and TB were relatively potent inducers of hepatic xenobiotic metabolism, whereas CC and PG had comparatively little effect. In addition, both CC and PG were markedly cytotoxic at concentrations of 100 μM in the culture medium (data not shown).

Previous studies in the rat and the results described in Section 6.1. have demonstrated that TB can induce hepatic CYP1A and CYP2B subfamily forms, whereas BHT is a known inducer of CYP2B and CYP3A forms. Because of the known *in vivo* hepatic effects of BHT and TB in the rat it was considered that effects on CYP forms would constitute useful biomarkers of effect of the food additives for the subsequent *in vitro* mixtures studies. To assess the effects of the food additives on CYP forms in cultured rat hepatocytes both CYP form mRNA levels and enzyme activities were determined. The

CYP form mRNA levels examined were CYP1A2, CYP2B1 and CYP3A1, whereas the two CYP-dependent enzyme activities examined comprised 7-ethoxyresorufin O-deethylase and 7-benzyloxy-4-trifluormethylcoumarin (BFC) O-debenzylase. Previous studies with rat hepatocytes cultured in a 96-well plate format have shown that 7-ethoxyresorufin O-deethylase is a good marker for induction of CYP1A forms, whereas BFC O-debenzylase is a good marker for induction of CYP1A and CYP2B forms (Donato et al., 1993; Price et al., 2000). To assess the functional viability of the rat hepatocyte preparations used in these studies, the effects of β-naphthoflavone (BNF), sodium phenobarbitone (NaPB) and pregnenolone-16α-carbonitrile (PCN) were also studied. Previous studies have demonstrated that BNF, NaPB and PCN can induce CYP1A, CYP2B and CYP3A forms, respectively, in rat liver (Maurel, 1996; Nims and Lubet, 1996; Parkinson, 2001). The cytotoxicty of the four food additives to cultured rat hepatocytes was also evaluated employing the MTT assay. The reference items for the MTT assay were two known cytotoxins, namely menadione and cadmium chloride (Stubberfield and Cohen, 1989; Zimmerman, 1978).

6.6.1. Cytotoxicity of food additives to rat hepatocytes

To assess the cytotoxicity of the food additives, rat hepatocytes were cultured for 72 hours with 0.5-200 μM BHT and TB, 0.5-50 μM CC and PG and 0.5-50 μM CC, 20 μM cadmium chloride and 50 μM menadione. The treatment of rat hepatocytes with 0.5-100 μM TB had little effect on MTT formazan production, whereas treatment with 200 μM TB reduced MTT formazan production to 77% of control (Table 31). The treatment of rat hepatocytes with 0.5-20 μM PG did not result in any decreases in MTT formazan production. While the mean MTT formazan production for four experiments in hepatocytes treated with 50 μM PG was similar to the controls, some variability between experiments was noted, with both increases and decreases in MTT formazan production being observed. The treatment of rat hepatocytes with 0.5-200 μM BHT and 0.5-20 μM CC had little effect on MTT formazan production (Table 32). However, treatment with 50 μM CC significantly reduced MTT formazan production to 30% of control. The treatment of rat hepatocytes with 20 μM cadmium chloride and 50 μM menadione resulted in marked cytotoxicity with MTT formazan production being significantly reduced to 1 and 2% of control, respectively (Tables 31 and 32).

6.6.2. Effect of food additives on CYP mRNA levels and enzyme activities in rat hepatocytes

To assess the effect of the food additives of CYP mRNA levels, rat hepatocytes were cultured for 72 hours with 2-200 μM BHT and TB, 0.5-20 μM CC and PG, 0.05, 0.2 and 2 μM BNF, 50, 200 and 500 μM NaPB and 0.5, 2, 5 and 20 μM PCN. The treatment of rat hepatocytes for 72 hours with 2-200 μM TB resulted in a concentration-dependent induction of CYP1A2 mRNA levels (Table 33). CYP1A2 mRNA levels were increased 8.9, 34.3 and 50.3 fold by treatment with 50, 100 and 200 μM TB, respectively. In contrast, the treatment of rat hepatocytes with 2-200 μM BHT, 0.5-20 μM PG and 0.5-20 μM CC did not result in any induction of CYP1A2 mRNA levels. The reference item BNF produced a concentration-dependent induction of CYP1A2 mRNA levels, producing 12.6, 55.5 and 172.2 fold increases at concentrations of 0.5, 0.2 and 2 μM, respectively (Table 34). In contrast neither 50-500 μM NaPB, nor 0.5-20 μM PCN had any marked effect on CYP1A2 mRNA levels.

The treatment of rat hepatocytes for 72 hours with 2-200 μM BHT resulted in a concentration-dependent induction of CYP2B1 mRNA levels (Table 33). CYP2B1 mRNA levels were increased 3.0, 5.7 and 8.0 fold by treatment with 50, 100 and 200 μM BHT, respectively. The treatment of rat hepatocytes with TB, PG and CC also produced small effects on CYP2B1 mRNA levels. CYP2B1 mRNA levels were increased 3.6, 1.9 and 2.6 fold by treatment with 100 μM TB, 20 μM PG and 20 μM CC, respectively. The reference item NaPB produced a marked induction of CYP2B1 mRNA levels, producing 25.7, 39.0 and 39.4 fold increases at concentrations of 50, 200 and 500 μM, respectively (Table 34). In contrast neither 0.05-2 μM BNF, nor 0.5-20 μM PCN had any marked effect on CYP2B1 mRNA levels.

The treatment of rat hepatocytes for 72 hours with 2-200 μM TB and 2-200 μM BHT produced only small increases in CYP3A1 mRNA levels (Table 33). CYP3A1 mRNA levels were increased 2.3 and 2.2 fold by treatment with 200 μM TB and 200 μM BHT, respectively. The treatment of rat hepatocytes with 0.5-20 μM PG and 0.5-20 μM CC had little effect on CYP3A1 mRNA levels. The reference item PCN produced a concentration-dependent induction of CYP3A1 mRNA levels, producing 21.0, 26.4, 31.5 and 44.4 fold increases at concentrations of 0.5, 2, 5 and 20 μM, respectively (Table 34). However, there was a marked variability between experiments in the magnitude of the

induction of CYP3A1 mRNA levels by PCN. While treatment with 0.05-2 μM BNF did not produce any induction of CYP3A1 mRNA levels, treatment with 50-500 μM NaPB produced 1.8 to 7.0 fold increases in CYP3A1 mRNA levels.

The treatment of rat hepatocytes for 72 hours with 0.5-50 μM TB did not result in any increase in 7-ethoxyresorufin O-deethylase activity, whereas treatment with 100 and 200 μM TB increased enzyme activity to 140 and 231% of control, respectively (Table 35). The treatment of rat hepatocytes with 0.5-200 μM BHT resulted in increases in 7-ethoxyresorufin O-deethylase activity at concentrations of 20 and 50 μM (Table 36). Unlike TB and BHT, the treatment of rat hepatocytes with 0.5-50 μM PG and 0.5-50 μM CC did not result in any marked increases in 7-ethoxyresorufin O-deethylase activity, with enzyme activity being reduced at high concentrations of both PG and CC (Tables 35 and 36). The treatment of rat hepatocytes with 200 and 500 μM NaPB and 0.2 and 2 μM BNF increased 7-ethoxyresorufin O-deethylase activity to 236, 280, 864 and 1004% of control, respectively (Tables 35 and 36).

The treatment of rat hepatocytes for 72 hours with 0.5-20 μM TB did not result in any increase in BFC O-debenzylase activity, whereas treatment with 50, 100 and 200 μM TB increased enzyme activity to 223, 310 and 399% of control, respectively (Table 35). The treatment of rat hepatocytes with 0.5 and 2 μM BHT did not result in any increase in BFC O-debenzylase activity, whereas treatment with 5, 20, 50, 100 and 200 μM BHT increased enzyme activity to 123, 173, 223, 244 and 189% of control, respectively (Table 36). Unlike TB and BHT, the treatment of rat hepatocytes with 0.5-50 μM PG and 0.5-50 μM CC did not result in any increases in BFC O-debenzylase activity, with enzyme activity being reduced at high concentrations of both PG and CC (Tables 35 and 36). The treatment of rat hepatocytes with 200 and 500 μM NaPB and 0.2 and 2 μM BNF increased BFC O-debenzylase activity to 716, 678, 223 and 270% of control, respectively (Tables 35 and 36).

6.7. Rat in vitro Studies with Mixtures of Food Additives

6.7.1. Design of rat in vitro mixtures study

The experimental design for the rat *in vitro* mixtures study with BHT, CC, PG and TB was provided by Dr Rob Stierum and Dr Eric Schoen. Table 37 shows the initially selected design with a control group (no compound treatment) and 30 experimental groups consisting of doses of one or more of the four food additives. For each of the four food additives the highest culture medium concentration was designated fractional dosage of 100 units, this being selected from the results of the rat *in vitro* studies with the individual food additives (see Section 6.6.). These concentrations were 100 µM for BHT, 15 µM for CC, 20 µM for PG and 100 µM for TB. In addition, a lower concentration of each food additive was also selected for study. These lower concentrations were 5 µM for BHT, 2 µM for CC, 2 µM for PG and 20 µM for TB. The experimental design for the four compound mixtures study as shown in Table 37 comprised the following combinations:

Group 0	Control
Groups 1 to 8	Single compound exposures (low and high concentrations)
Groups 9 to 14	Binary mixtures (high concentrations only)
Group 15 to 30	Quaternary mixtures (low and high concentrations)

When the rat in vitro mixture study was commenced some cytotoxicity was observed in the first two experiments (see Section 6.7.2.). The cytotoxicity to rat hepatocytes was most pronounced with the quaternary mixtures containing the highest 15 µM concentration of CC (Groups 23 to 30 in Table 37). In subsequent experiments a revised experimental design was adopted. Table 38 below shows the revised design with a control group (no compound treatment) and 31 experimental groups. In this design the quaternary mixtures containing the highest concentration of 15 µM CC (Groups 23 to 30) were deleted and replaced by quaternary mixtures containing 7.5 µM CC (Groups 33 to 40). The effect of 7.5 µM CC alone (Group 31) was also studied. The revised design shown in Table 38 thus comprised:

Group 0	Control
Groups 1 to 8	Single compound exposures (low and high concentrations)
Group 31	Single compound exposure (7.5 µM CC)
Groups 9 to 14	Binary mixtures (high concentrations only)

Group 15 to 22	Quaternary mixtures (employing 2 µM CC)
Group 33 to 40	Quaternary mixtures (employing 7.5 µM CC)

Table 38. Revised design of rat *in vitro* mixtures study with BHT, CC, PG and TB

Group[a]	Food additive concentration in culture medium (µM)			
	BHT	TB	PG	CC
Control	0	0	0	0
1	0	0	0	2
31	0	0	0	7.5
2	0	0	0	15
3	0	0	2	0
4	0	0	20	0
5	5	0	0	0
6	100	0	0	0
7	0	20	0	0
8	0	100	0	0
9	100	100	0	0
10	100	0	20	0
11	100	0	0	15
12	0	100	20	0
13	0	100	0	15
14	0	0	20	15
15	5	20	2	2
16	5	100	2	2
17	100	20	2	2
18	100	100	2	2
19	5	20	20	2
20	5	100	20	2
21	100	20	20	2
22	100	100	20	2
33	5	20	2	7.5
34	5	100	2	7.5
35	100	20	2	7.5
36	100	100	2	7.5
37	5	20	20	7.5
38	5	100	20	7.5
39	100	20	20	7.5
40	100	100	20	7.5

[a]Groups 1 to 8 and 31 are individual compound concentrations.
Groups 9 to 14 are binary mixtures (high concentrations only).
Groups 15 to 22 and 33 to 40 are quaternary mixtures (low and high concentrations of BHT, PG and TB, with revised concentrations of 2 and 7.5 µM CC).

6.7.2. Cytotoxicity of food additive mixtures to rat hepatocytes

Rat hepatocytes were cultured for 72 hours in control medium and medium containing the individual food additives and mixtures of food additives as shown in Table 37. Hepatocyte cultures were also treated with two known cytotoxins, namely 20 µM cadmium chloride and 50 µM menadione. The treatment of rat hepatocytes with the food additive mixtures shown in Table 37 resulted in some cytotoxicity which was most pronounced with the quaternary mixtures containing the highest 15 µM concentration of CC (Groups 23 to 30 in Table 37). Data for four experiments is shown in Table 39. While treatment with the first quaternary mixture containing 15 µM CC (Group 23) reduced MTT formazan production to 70% of control treatment with the other quaternary mixtures (Groups 24 to 30) significantly reduced MTT formazan production to 22-53% of control. As described in Section 6.7.1., the experimental design was modified to that shown in Table 38. The treatment of rat hepatocytes with the majority of the individual food additives and food additive mixtures shown in Table 38 had no significant effect on MTT formazan production (Table 40). MTT formazan production was significantly increased by one binary mixture (Group 10), but significantly decreased by one binary (Group 14) and one quaternary (Group 40) mixture. The treatment of rat hepatocytes with 20 µM and 50 µM menadione significantly reduced MTT formazan production to 2% of control (Table 40).

6.7.3. Effect of food additive mixtures on rat CYP mRNA levels and enzyme activities

The biomarkers selected for the rat *in vitro* mixtures study were levels of CYP1A2 and CYP2B1 mRNA, together with BFC O-debenzylase activity. Rat hepatocytes were cultured for 72 hours in control medium, medium containing the individual food additives and food additive mixtures shown in Table 38 and in medium containing known CYP inducers, namely 0.05, 0.2 and 2 µM BNF and 25, 100 and 250 µM NaPB. Each 96-well plate contained three or four controls (i.e. medium with 0.1% (v/v) DMSO only), with each individual food additive and food additive mixture being assayed in duplicate, with the replicates being located on different 96-well plates. A total of four experiments with different rat hepatocyte preparations were performed and the results for effect of CYP1A2 and CYP2B1 mRNA levels and BFC O-debenzylase activity summarised in Tables 41, 42 and 43, respectively.

The treatment of rat hepatocytes with 100 µM TB (Group 8 in Table 38) markedly increased CYP1A2 mRNA levels (Table 41), with increases also being observed in food additive mixtures containing 100 µM TB (Groups 9, 12, 13, 16, 18, 20, 22, 34, 36, 38 and 40). In contrast to the effect on CYP1A2 mRNA levels, the treatment of rat hepatocytes with either 100 µM BHT or 100 µM TB had no marked effect on CYP2B1 mRNA levels (Table 42). However, the treatment of rat hepatocytes with 100 µM BHT and 100 µM TB (Groups 6 and 8) increased BFC O-debenzylase activity as shown below in Table 43. BFC O-debenzylase activity was also affected by a number of the food additive mixtures, with both increases and decreases in enzyme activity being observed.

The functional viability of the rat hepatocyte preparations used for these studies was confirmed by the effects of the known CYP inducers BNF and NaPB. The treatment of rat hepatocytes with 0.05, 0.2 and 2 µM BNF produced a concentration dependent induction of CYP1A2 mRNA levels, whereas treatment with 25, 100 and 250 µM NaPB produced a concentration-dependent induction of CYP2B1 mRNA levels (Table 44). In contrast BNF had no marked effect on CYP2B1 mRNA levels and NaPB had no marked effect on CYP1A2 mRNA levels. Treatment with both BNF and NaPB resulted in an induction of BFC O-debenzylase activity.

The data shown in Tables 41, 42 and 43 was used for the statistical analysis of the effects of mixtures of food additives in rat hepatocytes (see Section 6.8.).

Table 43. Effect of treatment of rat hepatocytes with individual food additives and food additive mixtures on BFC O-debenzylase activity

Group[a]	Hepatocyte experiment				Enzyme activity[b]	% of control
	A	B	C	D		
Control	21	22	46	45	34 ± 7	(100)
1	22	21	45	36	31 ± 6	92
31	14	16	38	35	26 ± 6	77
2	17	21	42	34	29 ± 6	85
3	21	16	43	32	28 ± 6	83
4	25	29	82	37	43 ± 13	128
5	20	21	79	45	41 ± 14	123
6	37	34	164	113	87 ± 31	258
7	25	20	51	41	34 ± 7	102
8	38	46	78	62	56 ± 9	166
9	26	21	62	49	39 ± 10	117
10	36	58	139	83	79 ± 22	235
11	8	14	50	48	30 ± 11	90
12	33	45	64	56	49 ± 7	147
13	21	26	53	43	36 ± 7	106
14	15	26	21	9	18 ± 4	53
15	19	19	62	40	35 ± 10	104
16	24	38	60	65	47 ± 10	140
17	7	10	55	68	35 ± 16	104
18	13	15	18	19	16 ± 2	48
19	36	41	57	38	43 ± 5	128
20	37	43	66	60	52 ± 7	153
21	24	18	42	41	31± 6	93
22	12	20	19	20	18 ± 3	53
33	10	7	42	29	22 ± 8	66
34	28	23	59	55	41 ± 9	122
35	7	14	21	30	18 ± 5	53
36	12	19	15	18	16 ± 2	47
37	33	31	56	41	40 ± 6	120
38	29	48	79	63	55 ± 11	163
39	5	35	6	6	13 ± 7	38
40	9	16	11	7	11 ± 2	32

[a]See Table 38 for food additive culture medium concentrations.
[b]BFC O-debenzylase activity in units of pmol/min/mg hepatocyte protein.
Results are presented as mean ± SEM of 4 replicate experiments (experiments A, B, C and D).

6.8. Statistical Analysis of the Effects of Mixtures of Food Additives in Rat Hepatocytes and Inference of Mixtures Effects

The data described in Table 41,42 and 43, derived from 4 separate experiments involving 4 different hepatocyte isolates were used to assess any interaction between BHT, CC, PG and TB. As indicated before, primary aim was to test for deviation of effect addition (without further detailed characterization of any interaction in terms of dose dependency) Below, the outcome is described for CYP1A2, CYP2B1 and BFC O-debenzylase activity is discussed. In Fig. 20, CYP1A2 mRNA expression levels of rat hepatocytes treated for 72 hours with BHT, CC, PG and TB, relative to control levels, are shown. The left part of this Figure displays the relative expression levels, observed in hepatocytes treated with individual additives. This is essentially a repetition of the data reported in Table 41, but now expressed as geometrical mean, relatively to control. In addition, the significance of dose dependencies is shown, together with information on the shape of the dose response (linear, quadratic, cubic), which is derived from best fitted dose response curves through the data. The fitted dose response curves for each of the individual additives are at the base of establishing the additivity surface equation used to calculate the predicted values for mixtures. Based on these data, the additivity surface for the natural log of CYP1A2 followed the equation: $\ln(CYP1A2)= -0.002027*[BHT]-0.0222*[CC]+0.1666*[PG]-0.008919*[PG]^2+0.06041*[TB]-0.0002756*[TB]^2$, with concentrations in μM. Using this equation, it is possible to calculate for each mixture the predicted absolute expression value using the concentrations employed. From these subsequent calculations can be made of predicted values relative to control which are displayed in Fig. 20 and Figures below for other parameters. Student T-test was performed to test for significant differences between predicted and measured relative expression levels for these mixtures. Concerning the effect of binary mixtures on CYP1A2 mRNA expression, for all quaternary mixtures containing BHT and TB at 100 μM, the actual measured CYP1A2 levels were significantly ~2-fold lower than predicted levels. This was also observed in two quaternary mixtures containing BHT at 100 μM and TB at 20 μM. TB at 100 μM as individual additive resulted in a >25-fold induction. BHT as individual additive itself did not have any effect. Interestingly this antagonistic effect was not observed in the mixture containing BHT and TB only, at 100 μM each. Thus, this effect appears to be dependent upon the presence of CC and PG as well in these mixtures. Therefore, it is likely that these antagonistic effects observed in quaternary mixtures with respect to CYP can not be attributed to binary interactions only. Statistical modelling indicated that for two binary mixtures, containing PG at 20 μM, the actual measured fold induction was at a

higher level than predicted, based on the additivity surface equation. However, the relative fold induction in these mixtures was only minor in comparison to control, (1.7 to 2.9 fold).

In Fig. 21 results are shown for CYP2B1 mRNA expression. Results are shown relative to control. With respect to the individual additives, only BHT significantly induced dose dependently CYP2B1 mRNA expression in rat hepatocytes, primarily at the highest dose level of 100 μM. The Additivity Surface Equation used to calculate the predicted values for mixtures was equal to: ln(CYP2B1)= 0.012*[BHT]-0.659+0.00843*[PG]+ 0.000442*[TB], with an indicator variable for CC which is present in equation if CC is present in mixture, absent in equation if CC is absent in mixture. In binary mixtures containing BHT at 100 μM, measured levels were lower than predicted. Primarily the addition of PG and TB at their highest dose levels of respectively 20 μM and 100 μM resulted in a significant ~2-fold lower actual expression level, in comparison to predicted levels. Thus, addition of other additives to BHT at 100 μM seems to suppress the CYP2B1 inducing effect of BHT. In the quaternary mixtures of BHT at 100 μM + CC at 7.5 μM + PG at 2 μM + TB at 20 μM and of BHT at 100 μM + CC at 7.5 μM + PG at 2 μM + TB at 100 μM a similar observation was made: lower measured levels, in comparison to predicted levels. In all quaternary mixtures containing BHT at 5 μM, measured levels were at least 2-fold higher than predicted. In particular, this effect was most proncounced in the mixture of BHT at 5 μM + CC at 7.5 μM + PG at 20 μM + TB at 100 μM, with ~5.3-fold higher levels than predicted. However, it needs to be mentioned that even though these statistical inferences indicated possible deviations from effect addition, the actual measured CYP2B1 induction levels in these mixtures, relative to control were relatively modest (1.5 to 3.5 fold).

Fig. 22 displays the results for BFC O-debenzylase activity, relative to control. Statistical inference of the data obtained from individual additives in terms of curve fitting indicated dose dependencies for each of these additives, with primarily effects induced by BHT and TB. Based on these data obtained from individual additives, the additivity surface equation modelling the predicted effects in mixtures was equivalent to: ln(BFC)= 0.00774*[BHT]-0.0682*[CC]+0.00377*$[CC]^2$-0.113*[PG] +0.00608*$[PG]^2$+ 0.00508*[TB], with concentrations in μM. Using this equation, it was found that in the

binary mixture of BHT and TB at 100 μM each, measured levels of BFC O-debenzylase activity were lower than expected under the assumption of effect addition. Thus, it appears that, even though these additives both induce BFC O-debenzylase activity at these concentrations, joint addition resulted in absence of enzyme induction, at these concentrations. A similar effect was observed when CC at 15 μM was added to BHT at 100 μM. Further, in all quaternary mixtures containing BHT at 100 μM, the effect of BHT was counteracted by addition of the other additives. This effect (magnitude of difference between predicted and measured) was most pronounced in quaternary mixtures containing, in addition to BHT at 100 μM also TB at 100 μM and less pronounced in quaternary mixtures containing, in addition to BHT at 100 μM also TB at 20 μM. This suggests that in quaternary mixtures, the deviation from effect addition with respect to BFC O-debenzylase activity is primarily due to the addition of TB to BHT. However, given the observed interactions between BHT at 100 μM + CC at 15 μM (and CC at 15 μM and PG at 20 μM, although these compounds themselves did not induce BFC O-debenzylase activity), combined two-compound interactions contributing to this effect can not be excluded.

6.9. Human in vitro Studies with Individual Food Additives

Human hepatocytes were cultured in a "sandwich" configuration and treated with BHT, CC, PG, TB and the reference compounds for 72 hours. The reference compounds consisted of known inducers of CYP forms (namely, BNF, NaPB and rifampicin (RIF)) and two known cytotoxic agents (namely cadmium chloride and menadione). All of the human hepatocyte preparations used in theses studies were supplied by UK HTB and the majority were confirmed as being obtained from Caucasian donors. Details of all the preparations used for the studies with the individual food additives and the food additive mixtures (i.e. Sections 6.9 and 6.10.) are shown in Table 45. A total of eleven preparations were used, with cell viabilities at isolation ranging from 76.0-84.2%. The ages of the six male donors ranged from 57 to 77 years, whereas the ages of the five female donors ranged from 60 to 71 years.

Previous studies conducted in the rat demonstrated that BHT and TB were relatively potent inducers of hepatic xenobiotic metabolism, whereas CC and PG had comparatively little effect. The focus of the present studies with cultured human

hepatocytes was thus on evaluating the effects of BHT and TB. Like sodium phenobarbitone (NaPB), BHT is an inducer of hepatic CYP2B forms in the rat (Groten et al., 2000; Nims and Lubet, 1996; Price et al., 2004). However, in humans rodent CYP2B inducers are considered to be potential inducers of hepatic CYP3A forms, particularly of CYP3A4 (Maurel, 1996; Parkinson et al., 2004; Pelkonen et al., 1998). While NaPB can induce several CYP forms in human liver, including CYP2A, CYP2B, CYP2C and CYP3A subfamily forms, the induction of CYP3A subfamily forms is most important because this CYP subfamily accounts for the largest amount of the total CYP content in human liver (Chang et al., 1997; Gerbal-Chaloin et al., 2001; Maurel, 1996; Parkinson, 2001; Parkinson et al., 2004; Pelkonen et al., 1998). For the present investigations, the effect of BHT on CYP3A forms was evaluated by measurement of CYP3A4 mRNA levels. Previous studies in the rat have demonstrated that TB can induce hepatic CYP1A and CYP2B subfamily forms (Price *et al*., 2004). For rodent CYP2B subfamily effects, TB was evaluated as an inducer of human hepatocyte CYP3A4 mRNA levels, whereas to assess the effects of TB on CYP1A forms in human hepatocytes, the effect of TB on CYP1A2 mRNA levels was evaluated. While TB induces both CYP1A1 and CYP1A2 mRNA levels in rat liver (Price et al., 2004), only CYP1A2 is normally constitutively expressed in human liver (Parkinson, 2001). Thus for the present investigations, the effect of TB on CYP1A forms was evaluated by measurement of CYP1A2 mRNA levels. Finally, the effect of TB and BHT on CYP2B6 mRNA levels was also investigated. While levels of CYP2B6 are relatively low in human liver, this CYP form is inducible by many drugs including NaPB and RIF (Chang et al., 1997; Gerbal-Chaloin et al., 2001).

6.9.1. Cytotoxicity of food additives to human hepatocytes

To assess the cytotoxicity of the food additives, human hepatocytes were cultured for 72 hours with 5-200 μM BHT and TB, 2-50 μM CC and 5-200 μM PG, 20 μM cadmium chloride and 50 μM menadione. The treatment of human hepatocytes with 5-200 μM BHT and TB had little effect on MTT formazan production (Table 46). While the treatment of human hepatocytes with 5-50 μM PG did not result in any decreases in MTT formazan production, treatment with 100 and 200 μM PG reduced MTT formazan production to 56 and 27% of control, respectively. The treatment of human hepatocytes with 2-20 μM CC had little effect on MTT formazan production (Table 46). However, treatment with 50 μM CC reduced MTT formazan production to 47% of control. The

treatment of human hepatocytes with 20 μM cadmium chloride and 50 μM menadione resulted in marked cytotoxicity with MTT formazan production being reduced to 2 and 3% of control, respectively (Table 46).

6.9.2. Effect of food additives on CYP mRNA levels and enzyme activities in human hepatocytes

To assess the functional viability of the human hepatocyte preparations used in these studies, the effect of known CYP inducers (namely BNF, NaPB and RIF) on CYP mRNA levels was evaluated. Previous studies have demonstrated that BNF can induce CYP1A forms in human hepatocytes, whereas compounds such as NaPB and RIF can induce CYP3A, CYP2B and other CYP subfamily forms (Chang et al., 1997; Gerbal-Chaloin et al., 2001; Maurel, 1996; Parkinson, 2001; Parkinson et al., 2004).

Human hepatocytes were cultured for 72 hours with 0.5-200 μM TB, 2-200 μM BHT, 5-200 μM PG and 2-100 μM CC. The reference item concentrations were 10 and 50 μM for BNF, 200 and 1000 μM for NaPB and 10 and 50 μM for RIF. The treatment of human hepatocytes for 72 hours with 2-200 μM TB resulted in a concentration-dependent induction of CYP1A2 mRNA levels (Table 47). CYP1A2 mRNA levels were increased 16.6, 30.8, 49.5 and 52.8 fold by treatment with 20, 50, 100 and 200 μM TB, respectively. In contrast, the treatment of human hepatocytes with 2-200 μM BHT, 5-200 μM PG and 2-100 μM CC produced only small effects on CYP1A2 mRNA levels. The reference item BNF produced a concentration-dependent induction of CYP1A2 mRNA levels, producing 88.8 and 129.4 fold increases at concentrations of 10 and 50 μM, respectively (Table 48).

The treatment of human hepatocytes for 72 hours with 0.5-200 μM TB and 2-200 μM BHT resulted in a concentration-dependent induction of CYP2B6 mRNA levels (Table 47). CYP2B6 mRNA levels were increased 6.0, 11.9 and 16.6 fold by treatment with 50, 100 and 200 μM TB, respectively, whereas CYP2B6 mRNA levels were increased 5.6, 11.4 and 12.8 fold, respectively, by the same concentrations of BHT. The treatment of human hepatocytes with 50 and 100 μM PG also appeared to produce some increases in CYP2B6 mRNA levels. In contrast, treatment with 2-100 μM CC did not appear to produce any increase in CYP2B6 mRNA levels. The reference items NaPB and RIF produced concentration-dependent increases in CYP2B6 mRNA levels (Table 48).

CYP2B6 mRNA levels were increased 6.6 and 21.0 fold by treatment with 200 and 1000 μM NaPB, respectively and 10.0 and 12.6 fold by treatment with 10 and 50 μM RIF, respectively

The treatment of human hepatocytes for 72 hours with 0.5-200 μM TB and 2-200 μM BHT produced concentration-dependent increases in CYP3A4 mRNA levels (Table 47). CYP3A4 mRNA levels were increased 2.4, 4.5 and 10.0 fold by treatment with 50, 100 and 200 μM TB, respectively whereas CYP3A4 mRNA levels were increased 7.2, 7.8 and 8.2 fold, respectively, by the same concentrations of BHT. The treatment of human hepatocytes with 5-200 μM PG and 2-100 μM CC had no marked effect on CYP3A4 mRNA levels. The reference items NaPB and RIF produced concentration-dependent increases in CYP3A4 mRNA levels (Table 48). CYP3A4 mRNA levels were increased 9.9 and 23.2 fold by treatment with 200 and 1000 μM NaPB, respectively and 27.4 and 67.6 fold by treatment with 10 and 50 μM RIF, respectively.

To confirm that the increase in CYP mRNA levels was associated with increased CYP-dependent enzyme activities, human hepatocytes were treated with the food additives and 7-ethoxyresorufin O-deethylase and testosterone 6β-hydroxylase activities determined. Previous studies have demonstrated that these two enzyme activities are good markers for CYP1A2 and CYP3A4, respectively, in human liver (Maurel, 1996; Parkinson, 2001; Parkinson et al., 2004). The treatment of human hepatocytes for 72 hours with 50-200 μM TB resulted in significant increases in 7-ethoxyresorufin O-deethylase activity (Table 49). Enzyme activity was also markedly increased by treatment with 10 μM BNF. In contrast to the effect of TB, the treatment of human hepatocytes with 20-200 μM BHT, 20 μM CC and 50μM PG had little effect on 7-ethoxyresorufin O-deethylase activity in human hepatocytes (Table 49). The treatment of human hepatocytes with 20 and 50 μM BHT and with 1000 μM NaPB and 50 μM RIF resulted in significant increases in testosterone 6β-hydroxylase activity. Enzyme activity in cells cultured in control medium and medium containing 20 and 50 μM BHT, 1000 μM NaPB and 50 μM RIF was 396 ± 87 (mean ± SEM of 3 experiments), 1038 ± 107, 1071 ± 86, 1614 ± 149 and 2575 ± 264 pmol/min/mg hepatocyte protein, respectively (all $p<0.01$, except 50 μM RIF $p<0.001$).

6.10. Human in vitro Studies with Mixtures of Food Additives

6.10.1. Design of human in vitro mixtures study

The experimental design for the rat *in vitro* mixtures study with BHT, CC, PG and TB was provided by Dr Rob Stierum and Dr Eric Schoen. Table 50 below shows the selected design with a control group (no compound treatment) and 27 experimental groups consisting of doses of one or more of the four food additives. For each of the four food additives the highest culture medium concentration was designated 100 units, this being selected from the results of the human *in vitro* studies with the individual food additives (see Section 6.9.). These concentrations were 75 µM for BHT, 7.5 µM for CC, 10 µM for PG and 75 µM for TB. In addition, two lower concentrations, comprising 25 and 50 units, of each food additive were also selected for study. These lower concentrations comprised 18.75 and 37.5 µM for BHT, 1.875 and 3.75 µM for CC, 2.5 and 5 µM for PG and 18.75 and 37.5 µM for TB. The experimental design for the four compound mixtures study as shown in Table 50 comprised the following combinations:

Group 0	Control
Groups 1 to 12	Single compound exposures (25, 50 and 100 units)
Groups 13 to 18	Binary mixtures with a total dosage of 50 units
Groups 19 to 24	Binary mixtures with a total dosage of 100 units
Group 25	A quaternary mixture with a total dosage of 25units
Group 26	A quaternary mixture with a total dosage of 50 units
Group 27	A quaternary mixture with a total dosage of 100 units

Table 50. Design of human *in vitro* mixtures study with BHT, CC, PG and TB

Group[a]	Food additive concentration in culture medium (µM)			
	BHT	TB	PG	CC
Control	0	0	0	0
1	18.75	0	0	0
2	37.5	0	0	0
3	75	0	0	0
4	0	18.75	0	0
5	0	37.5	0	0
6	0	75	0	0
7	0	0	2.5	0
8	0	0	5.0	0
9	0	0	10	0
10	0	0	0	1.875
11	0	0	0	3.75
12	0	0	0	7.5
13	18.75	18.75	0	0
14	18.75	0	2.5	0
15	18.75	0	0	1.875
16	0	18.75	2.5	0
17	0	18.75	0	1.875
18	0	0	2.5	1.875
19	37.5	37.5	0	0
20	37.5	0	5.0	0
21	37.5	0	0	3.75
22	0	37.5	5.0	0
23	0	37.5	0	3.75
24	0	0	5.0	3.75
25	4.6875	4.6875	0.625	0.46875
26	9.375	9.375	1.25	0.9375
27	18.75	18.75	2.5	1.875

[a]Groups 1 to 12 are the individual compound concentrations (25, 50 and 100 units).
Groups 13 to 18 are binary mixture combinations comprising 25 units of each food additive.
Groups 19 to 24 are binary mixture combinations comprising 50 units of each food additive.
Groups 25, 26 and 27 are quaternary mixtures comprising 6.25, 12.5 and 25 units, respectively, of each food additive.

6.10.2. Cytotoxicity of food additive mixtures to human hepatocytes

Human hepatocytes were cultured for 72 hours in control medium and medium containing the individual food additives and mixtures of food additives as shown in Table 50. Hepatocyte cultures were also treated with two known cytotoxins, namely 20 µM cadmium chloride and 50 µM menadione. The treatment of human hepatocytes with the individual food additives and food additives mixtures shown in Table 50 had no

marked effect on MTT formazan production (Table 51). In contrast, treatment with 20 µM cadmium chloride and 50 µM menadione significantly reduced MTT formazan production to 2 and 4% of control, respectively (Table 51).

6.10.3. Effect of food additive mixtures on human CYP mRNA levels

The biomarkers selected for the human *in vitro* mixtures study were levels of CYP1A2, CYP2B6 and CYP3A4 mRNA. Human hepatocytes were cultured for 72 hours in control medium, medium containing the individual food additives and food additive mixtures shown in Table 50 and in medium containing known CYP inducers, namely 10 and 50 µM BNF, 200 and 1000 µM NaPB and 10 and 50 µM RIF. Each 96-well plate contained three or four controls (i.e. medium with 0.1% (v/v) DMSO only), with each individual food additives and food additive mixture being assayed in duplicate, with the replicates being located on different 96-well plates. A total of four experiments with different human hepatocyte preparations were performed and the results for effect of CYP1A2, CYP2B6 and CYP3A4 mRNA levels summarised in Tables 52, 53 and 54, respectively.
The treatment of human hepatocytes with 18.75, 37.5 and 75 µM TB (Groups 4, 5 and 6 in Table 50) markedly increased CYP1A2 mRNA levels (Table 52), with increases also being observed in food additive mixtures containing 18.75 and 37.5 µM TB (Groups 13, 16, 17, 19, 22 and 23). The treatment of human hepatocytes with TB also increased CYP2B6 mRNA levels (Table 53), whereas none of the food additives had any marked effect on CYP3a4 mRNA levels (Table 54).

The functional viability of the human hepatocyte preparations used for these studies was confirmed by the effects of the known CYP inducers BNF, NaPB and RIF. The treatment of human hepatocytes with 10 and 50 µM BNF produced a concentration dependent induction of CYP1A2 mRNA levels (Table 55). Levels of CYP2B6 and CYP3A4 mRNA were increased by treatment with 200 and 1000 µM NaPB and by 10 and 50µM RIF.

The data shown in Tables 52, 53 and 54 showing the results of four separate experiments was used for the statistical analysis of the effects of mixtures of food additives in human hepatocytes (see Section 6.11.).

6.11. Statistical Analysis of the Effects of Mixtures of Food Additives in Human Hepatocytes and Inference of Mixtures Effects

The data described in Table 52, 53 and 54, derived from 4 separate experiments involving 4 different hepatocyte isolates were used to statistically infer any interaction between BHT, CC, PG and TB. As indicated before, primary aim was to test for deviation of effect addition (without further detailed characterization of any interaction in terms of dose dependency). Below, the outcome is described for CYP1A2, CYP2B6 and CYP3A4 mRNA expression is discussed, in a similar manner as done for rat hepatocytes outlined in Section 6.8. Given the limited availability of sufficient amounts of human hepatocytes compared to rat hepatocytes and necessity to include positive controls (β-naphthoflavone, phenobarbitone and rifampicin) to check for donor responsiveness, in terms of Phase I metabolism, the design was confined to 27 dosage groups together with one control group. Emphasis therefore was on the effects of binary mixtures, together with three quaternary mixtures, in which additives were applied at low, mid and high dose levels. Figure 23 displays the outcome of statistical inference for mixture effects for human hepatocytes treated with additives using CYP1A2 mRNA expression as parameter. With respect to the effects of additives as individual compounds, TB clearly induced a dose dependent effect on CYP1A2 mRNA expression. Statistical inference indicated significant dose dependent effects for CC and PG as well, however from a biological perspective these have to be considered as minor. With respect to modelling mixture effects, the additivity surface equation, based on curve fittings from individual additives, was equivalent to: $\ln(CYP1A2) = -0.000705735*[BHT]+0.0313*[CC]+0.113*[PG]-0.0136*[PG]^2+0.280*[TB]-0.00670*[TB]^2 +4.98453^{-5}*[TB]^3$, with concentrations in μM. The major observation from comparison of prediction of CYP1A2 induction from this equation, with actual measured data was that PG significantly suppressed the effect of TB on CYP1A2 in binary mixtures, about half-fold. Interestingly, this finding was absent in the binary mixture of PG and TB for rat hepatocytes (Fig. 20). All binary mixtures tested of BHT and PG, and CC and PG, respectively showed deviation from effect addition, with measured levels higher than expected. However, in light of the magnitude of fold induction relative to control induced by TB and β-naphthoflavone as positive control (Table 55), these statistically inferred interactions probably need to be considered as less relevant from a biological perspective. For the quaternary mixture of BHT at 18.75 μM +

CC at 1.88 μM + PG at 2.5 μM + TB at 18.75 μM it was found that predicted CYP1A2 induction relative to control was higher than measured. The antagonistic effect in the quaternary was probably not due to any of the binary interactions. In contrast, the same mixture at low concentrations showed the opposite: lower predicted levels of CYP1A2, compared to measured (Fig. 23).

With respect to CYP2B6 mRNA expression statistical inference of the data indicated dose dependent effects for BHT, CC and clearly for TB (Fig. 24). The statistically inferred effects of BHT and CC have to be considered as minor in light of the effects induced by TB as well as the positive controls (Table 55, sodium phenobarbitone and rifampicin). The following equation describes the additivity surface from which predictions can be made with respect to the effects of mixtures on CYP2B6 expression: $\ln(CYP2B6)=0.0265*[BHT]-0.000186*[BHT]2-0.4478*[CC] +0.2003*[CC]^2-0.0177*[CC]^3+0.0125*[PG] +0.1333[TB]-0.00282*[TB]^2+2.02^{-5}[TB]^3$, with concentrations in μM. The most clear observation was that antagonism was observed for binary mixtures of BHT and TB: significant lower measured levels of CYP2B6, compared to predicted. Thus, even though BHT by itself did not have major effects on CYP2B6 induction, this additive seems to counteract the induction caused by TB, when given in a mixture. Within dose ranges tested, PG and CC displayed similar effects on TB-induced CYP2B6 expression, at least in the PG at 2.5 μM + TB at 18.75 μM, and CC at 3.75 μM + TB at 37.5 μM, respectively, dose groups. Statistical inference for deviation from effect addition indicated that the addition of BHT or CC at low dose levels (18.75 and 1.88 μM, respectively) to PG at its lowest dose level of 2.5 μM, resulted in measured levels that were higher than predicted. However, this effect was only modest, and the magnitude of these effects was at or slightly around CYP2B6 mRNA levels observed in control cultures. Therefore, from a biological perspective, this finding is probably of less importance. No indications for mixture effects were observed for quaternary mixtures tested.

Figure 25 shows predicted and measured CYP3A4 data relative to control. Statistical inference indicated dose dependencies for these additives, primarily for TB. However the induction of CYP3A4 by BHT and CC relative to control needs to be considered as only modest. The equation used to model the additivity surface was: ln(CYP3A4)=-

$0.0322*[BHT]+0.0019*[BHT]^2-1.77^{-5}*[BHT]^3+0.0519*[CC]$ $+0.1303*[PG]-0.0114*[PG]^2+$ 0.0166[TB], with concentrations in μM. With respect to the binary mixtures, data indicated that addition of PG and CC at their mid dose levels of 5 μM and 3.75 μM respectively, to TB at 37.5 μM resulted in significantly lower measured levels than predicted. In all binary mixtures containing BHT at 18.75 μM, measured levels were slightly higher than predicted. However, the magnitude of these effects was close to levels observed in control cultures and therefore probably of minor importance. The same holds true for a binary mixture of CC at 3.75 μM and PG at 5 μM, for which statistical inference suggested antagonism. No indications for possible interactions with respect to induction of CYP3A4 mRNA levels were observed with quaternary mixtures tested in human hepatocytes.

7. DISCUSSION

The aim of the present project was to assess the joint actions and interactions between four food additives with the liver a target organ. The four food additives selected for this project were BHT, CC, PG and TB, as for these compounds the ILSI-Europe Acceptable Daily Intake Task Force concluded that combined actions or interactions might occur under experimental conditions (Groten et al., 2000). Studies were performed both in the rat *in vivo* and in cultured rat and human hepatocytes *in vitro*. Aim was to provide insight into the likelihood of interactions between these additives in man. Therefore, the following approach was followed:

- To obtain physiological findings (body weight liver weight, clinical chemistry etc.) from Sprague-Dawley rats exposed to individual additives for 28 days
- To correlate molecular findings based upon RT PCR, enzyme assays for several CYP and GST and toxicogenomics derived markers, with physiological endpoints Without the claim to resolve full causal relationships between the markers observed and the physiological endpoints explored; this would have required a different experimental set-up compared to the descriptions in the Scope of Work (e.g. involving studies using knock-out models (in mice) following the initial wild type studies). As far as possible the gene expression findings, in particular for the single compound exposure studies, are discussed below in relation to clinical and pathological endpoints.
- To transfer these molecular markers to the subsequent in vivo rat and human hepatocyte mixture studies, together with physiological endpoints
- To apply these markers as bridging markers to relate the in vivo findings towards observations in hepatocytes in vitro, first in rat and subsequently in human hepatocytes in vitro to allow for interspecies extrapolations

Initial range finding studies were conducted in rats given the four food additives in the diet for 28 days. The aim of these initial studies was to identify suitable compound dose levels and biomarkers of effect for subsequent mixtures studies. The effects of the four food additives on liver weight, serum clinical parameters, phase I and II enzyme mRNA and protein expression and activity were assessed. Furthermore, cDNA microarray analysis was performed to discover possible new biomarkers for mixture toxicology studies. At the dose levels examined in this study none of the four food additives examined appeared to be markedly hepatotoxic, as no marked effects were observed on a number of serum clinical chemistry parameters. Of the four

food additives studied, the most marked effects on the parameters of hepatic xenobiotic metabolism measured were produced by BHT and TB.

In the present study BHT was shown to be an inducer of CYP2B forms in rat liver as demonstrated by the effects on 7-pentoxyresorufin O-dependentylase activity, CYP2B1/2 apoprotein levels, CYP2B1 and CYP2B1/2 mRNA levels and the transcriptomics data. These data are consistent with the fact that CYPs are primarily regulated at the transcriptional level since at all biological levels, starting at the mRNA level, induction of CYP 2B1/2 was observed. The comparatively small increase in 7-ethoxyresorufin O-deethylase activity produced by BHT treatment is likely to be attributable to induction of CYP2C forms (Nims and Lubet, 1996), rather than of CYP1A forms. Indeed, the transcriptomics data demonstrated that BHT also induced CYP2C6 gene expression and there was no evidence of induction of either CYP1A1 or CYP1A2 apoproteins. The biochemical and transcriptomics studies also demonstrated that BHT can induce CYP3A and GST forms (e.g. GST mu type 2) in rat liver, as previously reported (Manson et al., 1997). As such, BHT appears to have similar properties to that of sodium phenobarbitone with respect to induction of CYP forms and phase II xenobiotic metabolising enzymes (Nims and Lubet. 1996; Okey, 1990). Overall, the present results with BHT are in agreement with the available literature for this compound (Manson et al., 1997; McFarlane et al., 1997; Sun and Fukuhara, 1997; Sun et al., 1996). A comparison of transcriptomics findings in relation to morphological changes in the liver suggested the following. Enlargement of the liver was evidently reflected by the increased absolute and relative liver weight, the latter already at 762 ppm. At the same dose level, microarray analysis indicated that CYP2B1/2 gene expression changes started to appear. Also, enlargement of hepatocytes, that is hepatocellular hypertrophy, together with enlargement of the nucleolus, was observed in the highest treatment group (10154 ppm). The observed hepatic hypertrophy probably reflected an increase of smooth endoplasmatic reticulum associated with increased enzyme activities, phenotypic features which have been associated with similar phenobarbital-induced hepatic effects. Next to mechanistic insights provided by microarray analysis, it appeared that, in terms of sensitivity, gene expression analysis was as sensitive for detecting these particular BHT effects as was relative liver weight, but certainly not at orders of magnitudes lower. In contrast, clear pathological findings as determined by light microscopy, were only observed at the highest dose level employed.

Previous studies have demonstrated that TB can induce CYP1A forms in cultured rat and human hepatoma cell lines and in cultured rabbit hepatocytes (Aix et al., 1994; Backlund et al., 1999;

Kikuchi et al., 1996; Rey-Grobellet et al., 1996). The present results confirm that TB can induce CYP1A forms in rat liver and also demonstrate that TB is an inducer of CYP2B forms and GST activities. The observation that TB is a mixed inducer of CYP1A and CYP2B forms in rat liver does not appear to have been previously reported in the literature and hence is a novel finding from these studies. With respect to CYP1A forms, TB appears to induce both CYP1A1 and CYP1A2 mRNA levels in rat liver. The transcriptomics data also demonstrated an increase in CYP1A2 after TB treatment. However, as demonstrated by the Western immunoblotting studies only CYP1A2 and not CYP1A1 apoprotein levels were induced in rat liver following TB treatment. The observed induction of 7-ethoxyresorufin O-deethylase activity by TB is thus attributable to the induction of CYP1A2. Like BHT, TB also induced CYP2B forms (as demonstrated by the effects on 7-pentoxyresorufin O-depentylase activity, CYP2B1/2 apoprotein levels and CYP2B1 and CYP2B1/2 mRNA levels) and a range of GST activities. The range of GST activities induced by TB included CDNB, which is a substrate for many but not all GST forms (Beckett and Hayes, 1993; Eaton and Bammler, 1999), as well as CH, DCNB, EA and ENPP which have been reported to be useful substrates for alpha, mu, pi and theta class GST forms, respectively (Eaton and Bammler, 1999; Mannervik et al., 1985; Sherratt and Hayes, 2002). However, it should be noted that none of these substrates are totally selective for any particular GST class and/or form. The present data demonstrates that TB can induce both CYP1A and CYP2B forms and a range of GST forms in rat liver.

The aim of the transcriptomics studies with the individual compounds was to identify possible novel biomarkers of effect of the food additives which could be employed in the subsequent mixtures studies. It should be stressed that the aim of these studies was, by any means, not to redefine the currently established NOAELS for these additives. Expression levels of five genes associated with p53 were found to change upon TB treatment. These included p53 itself (~2-fold up); growth arrest and damage inducible protein 45α (GADD45α) (~1.7-fold up); neuronal cell death related gene in neuron-7 (DN-7) (1.5-1.9-fold down), the protein kinase C β (1.7-fold up) and serum albumin (~2-fold down). p53 is a well known tumour suppressor gene (Levine et al. 1994), related to G1/S-phase cell cycle control, DNA damage processing and apoptosis. Several downstream genes for p53 have been identified, e.g. p21(waf1/cip1), mdm2 and GADD45. GADD45 is transcriptionally upregulated under conditions were p53 protein levels are increased with oxidative stress (Kastan, Zhan, el Deiry, Carrier, Jacks, Walsh, Plunkett, Vogelstein, & Fornace, Jr. 1992;Zhan, Bae, Kastan, & Fornace, Jr. 1994). It is also known that p53 induction by oxidative stress is mediated through oxidative DNA damage. This generated the hypothesis

that administration of TB at very high dose levels alters p53 dependent pathways in the rat, through induction of oxidative DNA damage. TB has been shown to induce oxidative stress (Delescluse et al. 2001) and indeed induction of p53 protein levels was observed, at least at the highest dose level employed, which would yield a ~50-fold higher intake than the current NOAEL for the rat. The alterations in DN-7 mRNA expression are further in line with alterations in p53 metabolism. DN-7 has been reported to influence the binding of mdm-2 towards p53 and hence influence its stabilisation. Moreover, Protein kinase C β has been both associated with induction of oxidative stress (Scivittaro, Ganz, & Weiss 2000) and p53 metabolism (Jiang et al. 2002). Further, several GST activities were upregulated upon TB treatment, suggesting the possibility for conjugation of reactive TB intermediates to glutathione. No direct clear association between these p53 related gene expression changes and pathology/physiology was observed. Slight increases in serum ALT and serum LDH were observed in some of the higher TB dose groups; however these were minor and not at all indicative for frank hepatotoxicity. Increases in ALT have been associated with hepatocellular apoptosis (Acar, Ceyhan, & Colakoglu 2006;Eum, Cha, & Lee 2007). Liver weights were decreased, however, no apoptosis was observed at the microscopic level. TB administration at the highest dose level caused a reduction in food intake, which explains the observed reduction in body weight gain (in the two highest dose level groups). Also, liver weights were reduced and histopathology indicated reduced hepatic glycogen content at the highest dose level. Together, this suggests that these pathological observations are primarily due to reduced food intake resulting in reduced glycogen content, and as a resultant decreased liver weight. An increased relative liver weight was observed in the two highest dose groups; however which was primarily due to a decrease in body weight. Ando et al. (Ando et al. 2002) have associated caloric restriction with alterations in p53 dependent apoptotic pathways in liver of F344 rats. Their study indicated that p53 mRNA expression and p53 positive hepatocytes were increased in 3 months old animals that were calorically restricted in comparison to controls, starting from 1.5 months of age. In contrast, prolonged caloric restriction resulted in the opposite: reduced p53 mRNA expression and a reduction in the activation of p53 downstream mediators of apoptosis. Therefore, reduced food intake, during sub chronic time periods (e.g. 28 days to 1.5 month) may be, next to a direct action by TB, an additional explanation for the activation of p53 dependent pathways upon administration of TB at high dose levels. With respect to the human situation, these p53 findings for TB need to be put into perspective. The use of TB is not permitted anymore for the surface treatment of citrus fruits, and not as true additive anymore (Directive

98/72/EC, 1998) (1998), hence intake of TB by humans is probably very low. The established NOAEL for TB was not based upon the present study and therefore comparisons are subject to interstudy variations. However, it can be mentioned that the theoretical maximum daily intake of TB would be estimated to be <10% of the ADI (Groten, Butler, Feron, Kozianowski, Renwick, & Walker 2000), thus more than 1000-fold lower than the current NOAEL in the rat. This would imply 50000-fold lower than the intake provided by the dose level of 5188 ppm, at which alterations were observed in p53 metabolism in the present rodent study. In conclusion, these p53 findings for TB are of little toxicological concern to man. Together with the observed minor increase in p53 protein levels in rat liver, this lead to the decision that p53 was not selected as subsequent bridging marker in in vivo mixture and in vitro hepatocyte studies.

In contrast to BHT and TB, treatment with CC and PG had comparatively little effect on the markers of hepatic xenobiotic metabolism measured. The transcriptomics data indicated that CC may produce peroxisome proliferation in rat liver. CYP4A3 is known to be inducible by peroxisome proliferators and enoyl-CoA hydratase is a component of the peroxisomal fatty acid β-oxidation cycle, which can be markedly induced by peroxisome proliferators (Ashby et al., 1994; Klaunig et al., 2003; Lake, 1995). However, CC only produced a small increase in hepatic palmitoyl-CoA oxidation at the two highest dose levels (CC4062 and CC10154) examined in the range finding study. Established NOAELs were not derived from the current experiments. However, it may be carefully concluded that the lowest dose group of CC4062 (actual 4165 ppm) at which peroxisome proliferation was observed, already had a >2-fold higher intake (460 mg/kg/day) than the established NOAEL for CC of 220 mg/kg/day. These results demonstrate that CC is only a very weak rodent peroxisome proliferator. In terms of sensitivity, gene expression analysis and palmitoyl-CoA oxidation proved to be more sensitive than light microscopy to detect these weak CC effects. Further, these changes were already observed in absence of effects of CC exposure on liver weight. Moreover, chemically-induced peroxisome proliferation in the rat with respect to human hazard is of less relevance. These findings for CC indicate that there is no toxicological concern to man. Together, the small increases produced at only high CC doses irrelevant to man, precluded the use of peroxisome proliferation endpoints as biomarkers for subsequent studies with mixtures of the food additives in vivo and in vitro in hepatocytes.

Treatment with CC had little effect on the CYP-dependent enzyme activities and GST activities determined. In some studies CC has been reported to inhibit CYP-dependent enzyme activities

both *in vitro* and *in vivo* (Mori et al., 2006; Oetari et al, 1996; Sharma et al., 2005; Singh et al., 1998; Thapliyal and Maru, 2001). CC has also been reported to have complex effects on GST forms with both enzyme induction and inhibition being reported (Iqbal et al., 2003; Oetari et al., 1996; Sharma et al., 2001, 2005; Singh et al., 1998; Suzan and Rao, 1992).

In the present study PG was found to have little effect on the markers of hepatic phase I and II xenobiotic metabolism studied. PG has been reported not to induce rat hepatic total CYP content, CYP-dependent enzyme activities and GST activity (Depner et al., 1982; Stewart and Boston, 1987). However, in other studies PG has been reported to induce rat hepatic epoxide hydratase, UGT and GST activities (Manson et al., 1997; Stewart and McCrary, 1987). Since only modest effects were observed for PG with respect to liver and body weight, the gene expression changes detected upon PG treatment were difficult to relate to physiological changes.

With respect to the effects of these individual additives in Sprague Dawley rats, it is noticeable that in this study, transcriptome alterations were already observed at dose levels were no obvious changes in clinical chemistry or pathology was observed. Detailed statistical modelling of dose dependency of gene expression data was beyond the scope of these studies. Further, established NOAELs were not derived from the present study, which makes detailed comparisons between microarray data and conventional data complicated and subject to possible interstudy variations (e.g. different strains and dosage regimens). However in terms of providing a first estimate of the sensitivity of gene expression analysis, in comparison to more conventional endpoints that underlie NOAELs and Effect Levels cited in Groten et al. (Groten et al,. 2000), the following preliminary conclusions can be drawn. For BHT, gene expression changes were in general not observed at dose levels around the reported NOAEL (254 ppm), but primarily at levels around or higher than the observed Effect Level (1015 ppm). For CC subtle gene expression changes occurred around the NOAEL (2234 ppm), with more profound changes at dose levels around the Effect Level (4468 ppm). For PG, obvious gene expression changes were generally only observable at dose levels higher than the Effect Level (1531 ppm). For TB, gene expression changes already occurred in general at the currently established NOAEL (102 ppm) with further changes at levels around or higher than Effect Level (376 ppm).

In conclusion, gene expression analysis for some of these additives occurred at dose levels certainly around but certainly not many orders of magnitude below the current NOAEL

(obtained from different studies though) for these individual additives. Some of these gene expression changes could be mechanistically associated with clinical endpoints, without claiming full causality however. It needs to be stressed clearly that the aim of these gene expression studies, together with targeted phase I (CYP) and phase II (GST) metabolism assays, was not to redefine the current NOAELs for these additives. Instead the aim was to:

- generated candidate markers which could be used to facilitate the in vivo towards in vitro towards subsequent interspecies extrapolation (which could not have been accomplished using only liver weight and body weight as endpoints, since these can not be obtained in vitro)
- Obtain mechanistic insights into the action of these additives
- Relate these, if possible, to changes occurring in clinical endpoints (e.g. liver weight, body weight, histopathology), if any.

Overall, the rat *in vivo* range finding studies with the four food additives identified some useful biomarkers for the subsequent rat *in vivo* mixtures study. Clearly both BHT and TB are inducers of phase I and II rat hepatic xenobiotic metabolising enzymes. The biomarkers selected for the *in vivo* rat mixtures study thus comprised total CYP content, 7-ethoxyresorufin O-deethylase and 7-pentoxyresorufin O-depentylase activities, CYP1A2 and CYP2B1 mRNA levels and GST activities towards CDNB and DCNB as substrates.

The purpose of the subsequent rat *in vivo* mixtures study was to investigate if joint actions or interactions occur between BHT, CC, PG and TB, with respect to the liver as target organ, with focus on binary and quaternary mixtures. The study design enabled the establishment of dose response curves for individual compounds, and from these inferred additivity surface equations for binary and quaternary mixtures tested. Using these equations it was possible to infer predicted responses for biomarkers mentioned above. Statistical comparison (Student t-test) of predicted with measured, that is actual, responses allowed for testing of compatibility of the data with classical concepts in mixture toxicology: effect addition and dose addition. If significant deviations from effect (and dose) addition were noticed, it was proposed that interactions between additives, with respect to the biomarker endpoint studied, were likely. Further, the interactions were, if present, characterized in more detail in terms of dose dependency.

Male Sprague-Dawley rats were exposed to diets containing the individual food additives and both binary and quaternary mixtures of the food additives for 28 days. Statistical dose-response modelling for the individual compounds tested within the mixture study, basically confirmed the majority of findings observed in the range finding study discussed above. Thus, for BHT, significant dose dependent effects were observed for total liver weight, relative liver weight, total CYP content, 7-pentoxyresorufin O-dependentylase, 7-ethoxyresorufin O-deethylase and GST activities and CYP2B1 induction (Table S-5, Fig. 15-Fig. 19). These observations, including the effects on CYP2B1 and GSTs, are in agreement with other studies (Manson et al., 1997b; Price et al., 2004; Sun and Fukuhara, 1997; Sun et al., 1996). With respect to other parameters, no BHT effects were observed. No significant dose-dependencies were observed with CC, except for a significant, but non dose-dependent, effect on CYP2B1 mRNA induction where levels were increased at only the CC25 dose level. Statistically significant dose dependencies were observed for PG on 7-pentoxyresorufin O-dependentylase, 7-ethoxyresorufin O-deethylase, GST activity towards DCNB and CYP1A2 (Fig. 15-Fig. 19**,** and Table S-5, S-12, S-13, S-14, S-16). However, these effects where modest in comparison to those produced by BHT and TB. With respect to the effects of TB, dose-dependency was observed for liver weight, CYP, 7-pentoxyresorufin O-dependentylase, 7-ethoxyresorufin O-deethylase and GST activities towards CDNB and DCNB as substrates and for CYP2B1 and CYP1A2 mRNA induction. The effects of TB on these parameters are in agreement with data from the range finding studies (Price *et al.* 2004).

Regarding the effects of mixtures, no interactive mixture effects were observed on body weight, except for minor effects invoked by binary mixtures of BHT and TB, BHT and PG and the quaternary mixture at total fractional dosage of 100 (Fig. 15 and Supplementary Statistics materials). Considering the effect of BHT and TB in greater detail, a significant but small difference was observed between predicted and measured body weights for the BHT_TB50 group. In contrast, no significant difference between body weights were observed for BHT_TB100 compared to expected levels. Therefore, this interaction effect can only be classified as modest from a biological perspective. PG and TB interacted in a modest antagonistic way with respect to body weight: measured levels in this group where 87% compared to control, relative predicted levels were 95%. However, no differences between expected and observed were found at the low dose binary mixture. The all 100 group, consisting of BHT, CC, PG and TB at fractional nominal dose levels of 25 each demonstrated increased

body weight compared to predicted, however at ~5% higher levels in comparison to control only. Therefore, the effects of binary and quaternary mixtures of additives on body weight are probably of no concern in terms of interaction and mostly follow simple effect addition.

Concerning liver weight, in most cases simple addition of effects was observed. However, with applied dosages of each of the individual additives in binary mixtures, no substantial changes in liver weights in comparison to control were observed. An exception is the quaternary group with fractional dosages of 25% each: the all 100 group, in which interaction was observed with measured liver weight being 122% of control and predicted liver weight being 108% of control. Given the fact that no interactions were observed in binary mixtures containing CC and PG, respectively, at least based on this biomarker –except for a non-significant interaction between BHT and CC-, this interaction is probably of less relevance from a biological perspective, and likely primarily due to the relative high dose levels of BHT and TB. In conclusion with respect to body weight and liver weight, no or only modest interactions were observed between BHT, CC, PG and TB. In most cases, effect addition was applicable.

Despite this, the rat *in vivo* study with food additive mixtures showed evident interactions for parameters related to phase I and phase II drug metabolism. Most profound observations were the antagonistic interactions observed for the induction of CYP1A2 mRNA expression in all binary and quaternary mixtures, except for binary mixtures of BHT and CC in which the interaction was modestly synergistic. For some binary mixtures, e.g. CC_PG50, this effect was already observed at relative low dose levels. Expression of CYP1A2 mRNA is under control of the Ah receptor. The Ah receptor translocates upon binding to an agonist towards the nucleus and heterodimerizes with the transcription factor ARNT. Subsequently, this heterodimer binds to the xenobiotic responsive element and alters expression of genes including CYP1A1 and CYP1A2 (Klaassen, 2001). The inactive form of the Ah receptor consists of a ligand binding subunit, a dimer of HSP90 and X-associated protein 2. From a mechanistic perspective some of these additives have been reported in several *in vitro* and *in vivo* models to affect factors in this pathway and/or CYP1A1 and CYP1A2 expression. CC affected molecular chaperone function involving HSP90 (Ali and Rattan, 2006; Wu et al., 2006). CC directly inhibited dioxin-mediated Ah receptor activation (Amakura et al., 2003), activated the Ah receptor with stimulation of nuclear translocation and Ahr-ARNT complex formation (Rinaldi et al., 2002) activated DNA binding of the complex towards the XRE of CYP1A1 (Ciolino et al., 1998), and also reduced 7-

ethoxyresorufin O-deethylase activity (Singh et al. 1998) and promoted direct degradation of ARNT (Choi et al. 2006). TB was found to utilize the Ah-ARNT-XRE receptor pathway for induction of CYP1A1 expression (Backlund et al., 1999; Delescluse et al., 2001) and, as observed in the present study, induced CYP1A2 mRNA expression in rat (Price et al., 2004). Although BHT primarily induces the CYP2B family, BHT was a modest inducer at high dose levels of both CYP1A1 and CYP1A2 mRNA (Price et al., 2004). Thus, it might be possible that indeed combinations of these additives may antagonistically affect the Ah receptor pathway leading to a relative suppression of CYP1A2 mRNA expression as observed in the present study. Concerning the outcome of 7-ethoxyresorufin O-deethylase measurements, however, no clear interactions, (except for a probably spurious interaction between BHT and TB) were observed at the level of enzyme activity, and certainly not antagonistic ones. Although 7-ethoxyresorufin is metabolized mainly by CYP2C6 in liver microsomes from untreated rats, it is a marker for induction of CYP1A forms as well (Burke et al., 1994). Therefore, it is likely that although these additives do demonstrate antagonism with respect to the level of regulation of CYP1A2 mRNA expression level, mixture effects are probably not further exerted at the level of enzyme activity. What is important to mention is that the observed antagonism for CYP1A2 mRNA expression for some of these additive, occurred in absence of interactions at the level of liver and body weight. Therefore, these mechanistic interactions do not cause a noticeable change in physiology which can already predicted by simple addition of effects caused by the individual additives upon 28 exposure in Sprague-Dawley rats.

With respect to phase II drug metabolism, both CDNB and DCNB data demonstrated for the majority of mixture groups, primarily those containing BHT, measured enzyme levels that were modest, but significantly higher than observed (Fig. 17, Fig. 18 and Table S-5, S-15 and S-16) and statistically inferred interactions were evident. With respect to total CYP content, 7-pentoxyresorufin O-depentylase and CYP2B1 mRNA, in general aside from a few modest interactions, responses observed in mixture groups did not largely exceed responses observed with individual additives (see supplementary data). In conclusion, the rat *in vivo* studies with binary and quaternary mixtures of food additives shows that in male Sprague-Dawley rats treated for 28 days, with respect to liver weight and body weight, no major interactions were present. In most instances effect addition was applicable with, any magnitude of change compared to control not exceeding the observations made with individual additives. However, clear interactions, at least based on the statistical models calculated, were observed in phase I

metabolism, in particular at the level of CYP1A2 mRNA induction with 4 to 14-fold lower expression than expected, as well as glutathione S-transferase enzyme activities. Given the differences between the present study and earlier studies underlying the establishment for individual additives of NOAELs, and from these inferred ADIs as safety level in man, detailed interpretation of the present findings towards human risk assessment is not trivial. However, from a hazard perspective, the rat *in vivo* study indicates that additional liver enlargement invoked by mixtures of these additives appears to be unlikely, even at high dose levels.

The above experimentation was performed to address mixture effects according to classical principles in mixture toxicology which involves statistical testing for compatibility of measured data with the concepts of effect addition and dose addition, which can only be done for a limited number of parameters. This approach has the disadvantage that only a limited part of biology is captured. Genomics instead, here embodied in the application of DNA microarrays, has the potential to provide a global overview of compound-induced molecular changes: the transcriptome. Transcriptome analysis in the mixture study was performed to obtain insights if the observations made with conventional endpoints could be substantiated by a novel approach in mixture toxicology: the use of transcriptome profiles to determine the most dominant effects of individual compound within mixtures. This has not been done before for mixtures of food additives and represents a complete novel approach to mixture toxicology. To understand mixture effects from a different perspective, gene expression profiling was performed on a limited number of liver samples obtained from the rat *in vivo* mixture study. This is a completely new approach to mixture toxicology and aims to infer any (dis)similarity in action between additives by comparison of the complete gene expression profiles derived from single compound and mixture exposures. Below, main gene expression findings are discussed, as far as possible in relation to the rat *in vivo* data pertaining to conventional endpoints (liver weight, body weight) and assays for phase I and phase II metabolism. Global gene expression analysis by means of clustering and multivariate statistics, as well as determination of number of differentially expressed genes compared to control, indicated that for single food additives, gene expression profiles for both dose levels (at fractional dose level of 50 and 100) were quite similar. However, for individual genes dose dependencies still may exist, indeed as was shown by RT-PCR for CYP1A2 (e.g.TB) and CYP2B1 (e.g. BHT). It is noteworthy that gene expression changes were noticed at dose levels where no significant effects on liver weight and body weight were observed.

Treatment with CC had the weakest effect on global transcriptome changes, compared to other additives, in line with the absence of dose dependent effects on body weight, liver weight, and Phase I and Phase II metabolism parameters. Both BHT and PG as individual additives had more pronounced effects on the hepatic transcriptome, in comparison to CC. This is also in line with the observations made for other endpoints. TB had the most profound effect on gene expression changes at a fractional dosage of 50.

Concerning the gene expression patterns in mixtures, the number of differentially expressed genes was larger in binary mixtures in comparison to individual additives at equivalent fractional dose levels, primarily in comparison of CC and PG. As for its action as an individual additive, TB also had the most dominant effect on gene expression in mixtures, primarily in binary combinations. Gene expression profiles obtained from binary mixtures containing TB were most distinct from control. Further, the number of additional new genes, specific for binary mixtures containing TB that could not already be attributed to the individual additives, was modest. This indicates that the intrinsic effect of TB on the gene expression pattern within mixtures is most dominant.

Addition of PG or CC to BHT did not further influence the transcriptome changes already induced by BHT to a large extent, at least within the dose region tested. Similar findings were obtained from the conventional endpoints, e.g. relative levels to control of CDNB activity for BHT_CC100 and BHT_PG100 were close to BHT50 and BHT100.

Gene expression findings for the binary mixture of CC_PG100, with each of the additives at fractional dose level of 50, were interesting. Several data analysis approaches indicated that, whereas each of the individual additives CC and PG at either fractional dose level of 50 or 100, did not induce profound changes in gene expression pattern, the hepatic transcriptome was substantially changed upon administration of this mixture. This may be indicative of a true mixture effect, not related to exposure to individual additives. Indeed, functional biological interpretation of the data by means of T-profiler analysis, (biological interpretation of array data was done in a limited way and additional to the Scope of Work submitted to FSA in April 2006) suggested the enrichment of genesets entitled ‘transcription’, ‘regulation of transcription’ and ‘sequence-specific DNA binding’, categories that were not enriched by CC and PG alone.

It needs to be mentioned however that these observations were observed in absence of any clinical relevant interactions. Evidently, within the scope of a 28 day dietary exposure scenario, Sprague-Dawley rats are fully capable of coping with joint exposures of CC and PG in terms of physiology: despite interactions at the molecular level as revealed by e.g. CYP1A2 mRNA expression and gene expression studies effects on liver and body weights are explained by simple addition of the effects invoked by the individual additives with resulting weights being close to control.

To allow for further possible *in vitro-in vivo* and interspecies extrapolations, studies were also performed with cultured rat and human hepatocytes.

A molecular marker-based approach was chosen to facilitate this. It is understood that, within the observational in vivo studies performed here, some of the changes in these molecular markers are difficult to reconcile in terms of their direct relation with clinical endpoints. However, such an approach was deemed as the only way to allow for interspecies extrapolation of potential effects of mixtures of additives, since the outcome of clinical endpoints, both for single compound and mixture studies, can not be directly extrapolated to rat and human hepatocytes.

Many studies have demonstrated that cultured hepatocytes can be reliably used to evaluate the effects of chemicals on CYP forms and other xenobiotic metabolising enzymes and to evaluate species differences in response (Coecke et al., 1999; Hewitt et al., 2007; Maurel, 1996; Parkinson et al., 2004). The aim of these investigations was to determine suitable *in vitro* biomarkers of effect and appropriate concentration ranges of the four food additives for subsequent rat and human hepatocyte mixture studies. The results of the MTT cytotoxicity studies demonstrated that TB and BHT were not markedly cytotoxic to rat and human hepatocytes, with PG producing some cytotoxicity at concentrations of around 50 μM or higher. CC was the most cytotoxic of the four food additives studied, with concentrations >20 μM producing marked toxicity in rat and human hepatocytes. Rat and human hepatocytes were cultured in a 96-well plate format. Because of the small amounts of tissue available for analysis, sensitive biomarkers were required for the *in vitro* studies. The biomarkers evaluated comprised CYP mRNA levels and CYP enzyme activities employing fluorimetric endpoints.

In keeping with the *in vivo* studies in the rat TB was found to be a potent inducer of CYP1A2 mRNA levels in cultured rat hepatocytes, whereas BHT induced CYP2B1 mRNA levels. Both BHT and TB induced the CYP1A/2B marker BFC O-debenzylase (Price et al., 2000) in cultured rat hepatocytes. Hence, these three endpoints were selected as biomarkers for the rat *in vitro* mixtures studies.

The effect of the four food additives on CYP forms in cultured human hepatocytes was also determined. In these studies the functional viability of the human hepatocyte preparations used was confirmed by the use of known inducers of human CYP forms, namely BNF, NaPB and RIF. In agreement with previous studies in human hepatocytes BNF induced CYP1A forms, whereas both NaPB and RIF induced CYP2B and CYP3A forms (Chang et al., 1997; Gerbal-Chaloin et al., 2001; Maurel, 1996; Parkinson, 2001; Parkinson et al., 2004; Price et al., 2007). Studies with cultured human hepatocytes demonstrated that TB was an inducer of CYP1A2 mRNA levels and TB was also shown to induce CYP1A-dependent 7-ethoxyresorufin O-deethylase activity. Apart from investigating the effect of the four food additives on CYP1A2 mRNA levels, the effects of the test compounds on CYP3A4 and CYP2B6 levels were also determined. In the rat the induction of CYP2B forms by NaPB and related compounds (e.g. BHT) is mediated through effects on the constitutive androstane receptor (CAR; Honkakoski and Negishi, 2000). While CAR is expressed in human liver, the induction of human hepatic CYP forms by NaPB and related compounds appears to be more due to effects on the pregnane X receptor (PXR), than on CAR (Holsapple et al., 2006; Moore et al., 2003). Other studies have shown that NaPB induces CYP3A and other CYP forms, including, CYP2A, CYP2B and CYP2C forms, in human hepatocytes, these effects being mediated through PXR, CAR and the glucocorticoid receptor (Gerbal-Chaloin et al., 2001; Madan et al., 2003; Parkinson et al., 2004; Wang et al., 2003). In the present study BHT was found to induce both CYP3A4 and CYP2B6 mRNA levels, while having little effect on CYP1A2 mRNA levels. The induction of CYP3A forms by BHT was confirmed by significant increases in human hepatocyte testosterone 6β-hydroxylase activity. Like BHT, the treatment of human hepatocytes with TB also resulted in an induction of CYP3A4 and CYP2B6 mRNA levels. The results of these investigations thus demonstrate that as previously observed in the rat (Price et al., 2004) TB is a mixed inducer of CYP forms in human liver. The observation that TB is a mixed inducer of CYP1A, CYP2B and CYP3A forms in human hepatocytes does not appear to have been previously reported in the

literature and hence is a novel finding from these studies. As with the studies with rat hepatocytes both CC and PG had no marked effect on the CYP form mRNA levels measured in human hepatocytes. The present results with CC are in agreement with studies in human hepatocytes by Gross-Steinmeyer et al. (2004) where 10-50 µM CC did not induce CYP1A2 mRNA levels and by Raucy (2003) where 5 µM CC did not induce CYP3A4 mRNA levels. The three endpoints were selected as biomarkers for the human hepatocyte mixtures studies comprised CYP1A2, CYP2B6 and CYP3A4 mRNA levels.

As for rats *in vivo*, food additives were also tested in combinations in mixtures in rat and human hepatocytes, using the biomarkers proposed above. Experimental designs and concentrations chosen were not exactly identical between the different situations, given experimental limitations, but this approach allowed for a limited parallelogram comparison of binary and quaternary mixture effects observed in the different *in vitro* models and *in vivo*. This is discussed below, with emphasis on binary mixtures of BHT and TB and quaternary mixtures. With respect to CYP1A2 mRNA, the following *in vitro-in vivo* and cross species comparisons can be made. Concerning the effects of individual additives in rats *in vivo*, rat *in vitro* and human *in vitro*, the most clear dose dependency was observed for TB. Whereas no major evidence was found for any interactive effects with respect to body weight and liver weight, in rats *in vivo*, clear antagonistic - being the effect was less than could be predicted from the additivity surface equations which were in turn based upon the statistical models describing single compound dose response curves- mixture effects were observed with respect to CYP1A2. Concerning the effects in binary mixtures *in vivo*, antagonistic effects with respect to CYP1A2 mRNA were, primarily detected in binary mixtures containing TB. This is not surprising given the inducing effects of TB already displayed as individual additive. However, this was not the case for rat hepatocytes *in vitro*, at least for the binary mixtures tested, no antagonism for CYP1A2 mRNA expression. Further, in human hepatocytes, antagonism was only observed for binary mixtures of PG and TB, interestingly the same combination of additives for which the largest deviation from effect addition was inferred from statistical modelling of the *in vivo* data (PG_TB100 group; Fig. 19). However, for the other binary mixtures containing TB, antagonism was absent *in vitro* in rat and man. In all quaternary mixtures tested *in vivo*, measured CYP1A2 mRNA levels were lower than predicted. Also, in line, in some of the quaternary mixtures in rat hepatocytes, measured CYP1A2 levels were lower than predicted. In the three quaternary mixtures tested in human hepatocytes, this was also the case in the mixture containing the highest dose levels of all

compounds. In conclusion, CYP1A2 mRNA expression appears to be a marker for effects of TB, and the antagonistic effects in high dose levels of quaternary mixtures of BHT, CC, PG and TB, across species and the *in vitro-in vivo* boundary. However, the *in vitro* and *in vivo* interspecies extrapolations for CYP1A2 mRNA induction induced by these additives in binary combinations are less clear. Concerning the outcome of 7ER measurements in vivo, no clear interactions were observed at the level of enzyme activity, and certainly not antagonistic ones. Although 7-ethoxyresorufin is metabolized mainly by CYP2C6 in liver microsomes from untreated rats, it is a marker for induction of CYP1A forms as well (Burke et al., 1994). Therefore, it might be that although these additives do demonstrate antagonism with respect to the level of regulation of CYP1A2 mRNA expression level, this is possibly not further exerted at the level of enzyme activity.

It is known that NaPB, an inducer of CYP2B in the rat, induces CYP3A4 through PXR and CYP2B6 through CAR in human liver. Further, rat CYP2B inducers also induce other CYP forms. In this study, TB was found to be a mixed inducer of CYP1A2 mRNA and CYP2B1 mRNA and BHT was a clear inducer of CYP2B1 mRNA in the rat. Further, both BHT and TB induced CYP3A4 and CYP2B6 mRNA in human hepatocytes. Therefore, it is sensible to attempt to make *in vitro* towards *in vivo* and interspecies extrapolations concerning mixture effects for CYP2B1 mRNA in rat, and CYP2B6 and CYP3A4 in human hepatocytes. This was done primarily for BHT and TB, since only reliable conclusions could be drawn for mixture effects of BHT and TB on CYP2B1 mRNA in rat *in vivo*. The effects of mixtures of other additives were difficult to address, given problems with modelling the data for CC and PG (see document Supplementary Statistics.doc). No interactions were likely to occur for binary mixtures of BHT and TB in rat *in vivo*, concerning CYP2B1 mRNA expression. Instead, the effects were additive (simple effect addition). In contrast, in rat hepatocytes *in vitro* (Fig 21), addition of TB at 100 μM lowered the CYP2B1 inducing effect of BHT at 100 μM by about two-fold. A similar observation was made for CYP2B6 in human hepatocytes: antagonism was observed for the binary mixtures of BHT and TB: significant lower measured levels of CYP2B6, compared to those predicted. Thus, even though BHT by itself did not have major effects on CYP2B6 induction, this additive seems to counteract the induction caused by TB, when given in a mixture. No such effects were observed for mixtures of BHT and TB on CYP3A4 mRNA in human hepatocytes. Together, concerning the effects of mixtures of BHT and TB on rat CYP2B1 mRNA *in vitro* and *in vivo*, jointly with CYP2B6 and CYP3A4 mRNA in human

hepatocytes *in vitro*, no clear *in vitro* to *in vivo* extrapolations could be made. Results for CYP2B6 mRNA in human hepatocytes were somewhat comparable to CYP2B1 mRNA in rat hepatocytes; 2-fold lower measured levels than could be predicted from the additivity surface equation based upon the individual responses of BHT and TB. With respect to the other mixtures the following can be mentioned. Although modest, in quaternary mixtures of BHT, CC, PG and TB, CYP2B1 mRNA levels in rat were approximately two-fold higher than expected (Fig. 21). In contrast, no indications for synergism were observed for CYP2B6 and CYP3A4 mRNA expression, for the three quaternary mixtures tested in human hepatocytes. In binary mixtures of CC and TB and PG and TB respectively, rat *in vitro* CYP2B1 mRNA levels were slightly higher than predicted, but still close to control. In contrast, for some of the binary mixtures of these compounds tested in human hepatocytes, CYP2B6 and CYP3A4 were lower than predicted. In conclusion, with respect to the effects of mixtures on NaPB-inducible CYPs such as CYP2B1, CYP2B6 and CYP3A4, *in vitro* towards *in vivo* extrapolation in rat was not clear from the data. Interspecies extrapolation in terms of comparison of data from rat and human *in vitro* provided a mixed answer: in some instances e.g. for binary mixtures of TB and BHT concordance between the *in vitro* models; with disconcordance for quaternary mixtures and binary mixtures of CC and TB, and PG and TB, respectively.

In rat hepatocytes, in a binary mixture of BHT and TB each at 100 μM, as well as all quaternary mixtures containing these additives at the same concentrations together with CC and PG, BFC O-debenzylase activity levels, as marker for both CYP1A and CYP2B activities, were substantially lower than predictions inferred from the additivity surface equation (Fig. 22). This was comparable to the effects of this mixture on CYP2B1 mRNA data for this mixture (lower than predicted), but not to CYP1A2 mRNA (no difference). Therefore, using BFC O-debenzylase activity as marker for mixture effects of food additives, in relation to transcriptional regulation of CYP1A2 and CYP2B1, is complicated.

Altogether, this study indicates that in Sprague Dawley rats *in vivo* major interactions between BHT, CC, PG and TB, administered for 28 days in the diet, with respect to clinical endpoints as body weight and liver weight are unlikely, at least for the binary and quaternary combinations tested. In most instances, simple effect addition was applicable. In contrast, antagonism was observed for CYP1A2 mRNA expression and modest synergism was observed for phase II metabolism, represented as GST activities towards CDNB and DCNB. Gene expression analysis

performed on liver samples obtained from the rat *in vivo* range finding studies with individual additives corroborated the mRNA expression findings for CYPs. Further, based upon the transcriptomics data, it was found that CC is a weak peroxisome proliferator and TB a weak inducer of p53 protein, however only at high dose levels likely irrelevant to man. Gene expression analysis also proved to be a useful method to identify the most dominant additive in these mixtures (TB) and to identify mixture-specific effects that were not noticed with biomarkers for CYP and GST mRNA expression and activity (for CC+PG). However, it needs to be stated clearly that these mixture effects at the molecular level were not associated with profound mixture effects at the physiological level (liver weight, body weight), since these were absent in this 28 day rodent study. An attempt was made to extrapolate the rat *in vivo* mixture effects along the interspecies and *in vitro-in vivo* parallelogram approach, using rat and human hepatocytes cultured *in vitro*. This proved to be difficult, and was at best possible for quaternary mixtures employing CYP1A2 mRNA expression as biomarker. Given differences between the present study and earlier studies underlying the establishment for individual additives of NOAELs, and from these inferred ADIs as safety level in man, detailed interpretation of the present findings towards human risk assessment is not trivial. However, from a hazard perspective, the rat *in vivo* study indicates that additional liver enlargement invoked by mixtures of these additives appears to be unlikely, even at high dose levels exceeding the established NOAEL. In contrast, interactions were observed at the level of Phase I and Phase II metabolism and transcriptomics indicated possible mixture effects, which however were insufficient to cause changes in physiology (liver weight, body weight) beyond the level of the effects already invoked by the individual additives.

8. ACKNOWLEDGEMENTS

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9. REFERENCES

Acar, T., Ceyhan, K., and Colakoglu, T. (2006). Inhibition of apoptosis prevents liver failure and improves survival rates after extensive hepatectomy in rats. Acta Chir Belg. 106(6), 696-700.

Aix, L., Rey-Grobellet, X., Larrieu, G., Lesca, P., Galtier, P., 1994, Thiabendazole is an inducer of cytochrome P4501A1 in cultured rabbit hepatocytes. Biochem. Biophys. Res. Commun. 202, 1483-1489.

Ando, K., Higami, Y., Tsuchiya, T., Kanematsu, T., and Shimokawa, I. (2002). Impact of aging and life-long calorie restriction on expression of apoptosis-related genes in male F344 rat liver. Microsc. Res. Tech. 59(4), 293-300.

Ashby, J., Brady, A., Elcombe, C.R., Elliott, B.M., Ishmael, J., Odum, J., Tugwood, J.D., Kettle, S., Purchase, I.F.H., 1994. Mechanistically-based human hazard assessment of peroxisome proliferator-induced hepatocarcinogenesis. Hum. Exptl. Toxicol. 13 (Suppl.2), S1-S117.

Ali, R. E., and Rattan, S. I. (2006). Curcumin's biphasic hormetic response on proteasome activity and heat-shock protein synthesis in human keratinocytes. Ann. N. Y. Acad. Sci. 1067, 394-399.

Amakura, Y., Tsutsumi, T., Sasaki, K., Yoshida, T., and Maitani, T. (2003). Screening of the inhibitory effect of vegetable constituents on the aryl hydrocarbon receptor-mediated activity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Biol. Pharm. Bull. 26(12), 1754-1760.

Backlund, M., Weidulf, L., Ingelman-Sundberg, M., 1999. Structural and mechanistic aspects of transcriptional induction of cytochrome P450 1A1 by benzimidazole derivatives in rat hepatoma H4IIE cells. Eur. J. Biochem. 261, 66-71.

Beckett, G.J., Hayes, J.D., 1993. Glutathione *S*-transferases: biomedical applications. Adv. Clin. Chem. 30, 281-380.

Baldi, P., and Long, A. D. (2001). A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes. Bioinformatics. 17(6), 509-519.

Bartosiewicz, M., Penn, S., and Buckpitt, A. (2001a). Applications of gene arrays in environmental toxicology: fingerprints of gene regulation associated with cadmium

chloride, benzo(a)pyrene, and trichloroethylene. Environ. Health Perspect. 109(1), 71-74.

Bartosiewicz, M. J., Jenkins, D., Penn, S., Emery, J., and Buckpitt, A. (2001b). Unique gene expression patterns in liver and kidney associated with exposure to chemical toxicants. J. Pharmacol. Exp. Ther. 297(3), 895-905.

Bliss, C.I., 1939**.** The toxicity of poisons applied jointly. Ann. Appl. Biol. 26, 585-615.

Boorsma, A., Foat, B. C., Vis, D., Klis, F., and Bussemaker, H. J. (2005). T-profiler: scoring the activity of predefined groups of genes using gene expression data. Nucleic Acids Res. 33(Web Server issue), W592-W595.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C., Gaasterland, T., Glenisson, P., Holstege, F. C., Kim, I. F., Markowitz, V., Matese, J. C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S., Stewart, J., Taylor, R., Vilo, J., and Vingron, M. (2001). Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat. Genet. 29(4), 365-371.

Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T., Mayer R.T. 1985. Ethoxy-, pentoxy-, and benzyloxyphenoxazones and homologues : a series of substrates to distinguish between different induced cytochromes P-450. Biochem. Pharmacol. 34, 3337-3345.

Burke, M.D., Thompson, S., Weaver, R.J., Wolf, C.R., Mayer, R.T., 1994. Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver. Biochem. Pharmacol. 48, 923-936.

Cassee, F.R., Groten, J.P., van Bladeren, P.J., Feron, V.J., 1998. Toxicological evaluation and risk assessment of chemical mixtures. Crit. Rev. Toxicol. 28, 73-101.

Cassee, F.R., Sühnel, J., Groten, J.R., Feron, V.J., 1999. The toxicology of chemical mixtures, in: Ballantyne, B., Marrs, T.C., Syversen, T. (Eds), General and Applied Toxciology. Macmillan Reference Limited, London, pp.899-908.

Chang, T.K.H., Yu, L., Maurel, P., Waxman, D.J., 1997. Enhanced cyclophosphamide and ifosfamide activation in primary human hepatocyte cultures: response to

cytochrome P-450 inducers and autoinduction by oxazaphoshorines. Cancer Res. 57, 1946-1954.

Choi, H., Chun, Y. S., Kim, S. W., Kim, M. S., and Park, J. W. (2006). Curcumin inhibits hypoxia-inducible factor-1 by degrading aryl hydrocarbon receptor nuclear translocator: a mechanism of tumor growth inhibition. Mol. Pharmacol. 70(5), 1664-1671.

Chomczynski, P., Mackey, K., 1995. Modification of the TRI Reagent™ procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. BioTechniques 19, 942-945.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal.Biochem. 162, 156-159.

Ciolino, H. P., Daschner, P. J., Wang, T. T., and Yeh, G. C. (1998). Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. Biochem. Pharmacol. 56(2), 197-206.

Coecke, S., Rogiers, V., Bayliss, M., Castell, J., Doehmer, J., Fabre, G., Fry, J., Kern, A., Westmoreland, C., 1999. The use of long-term hepatocyte cultures for detecting induction of drug metabolising enzymes: the current status. ECVAM Hepatocytes and Metabolically Competent Systems Task Force Report 1. ATLA 27, 579-638.

Conesa, A., Heijne, W., van Erk, M., van der Lende, T., van Sonsbeek, H., Bouman, M., Kistemaker, C., Bausch, S., and van Ommen, B. (2001). TN-KnowBase: Expression, Analysis and Interpretation in Nutrigenomics. CHI's 2001 Genome Tri-Conference: Bioinformatics and In Silico Biology. Conference Book (Abstract).

COT, 2002. Risk Assessment of Mixtures of Pesticides and Similar Substances. Available at http://cot.food.gov.uk/pdfs/reportindexed.pdf.

Delescluse, C., Ledirac, N., Li, R., Piechocki, M. P., Hines, R. N., Gidrol, X., and Rahmani, R. (2001). Induction of cytochrome P450 1A1 gene expression, oxidative stress, and genotoxicity by carbaryl and thiabendazole in transfected human HepG2 and lymphoblastoid cells. Biochem. Pharmacol. 61(4), 399-407.

Depner, M., Kahl, G.F., Kahl, R., 1982, Influence of gallic acid esters on drug-metabolizing enzymes of rat liver. Food Chem. Toxicol. 20, 507-511.

Donato, M.T., Gómez-Lechón, M.J., Castell, J.V, 1993. A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. Anal. Biochem. 213, 29-33.

Eaton, D.L, Bammler, T.K., 1999. Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol. Sci. 49, 156-164.

Edwards, R.J., 1998. Targetting antipeptide antibodies towards cytochrome P450 enzymes, in: Phillips, I.R., Shephard, E.A. (Eds.), Methods in Molecular Biology, Vol. 107: Cytochrome P450 Protocols. Humana press, Totowa, pp. 239-249.

Eum, H. A., Cha, Y. N., and Lee, S. M. (2007). Necrosis and apoptosis: sequence of liver damage following reperfusion after 60 min ischemia in rats. Biochem. Biophys. Res. Commun. 358(2), 500-505.

Gerbal-Chaloin, S., Pascussi, J.-M., Pichard-Garcia, L., Daujat, M., Waechter, F., Fabre, J.-M., Carrère, N., Maurel, P., 2001. Induction of CYP2C genes in human hepatocytes in primary culture. Drug Metab. Dispos. 29, 242-251.

Gray, T.J.B., Lake, B.G., Beamand J.A., Foster J.R., Gangolli, S.D., 1983. Peroxisome proliferation in primary cultures of rat hepatocytes. Toxicol. Appl. Pharmacol. 67, 15-25.

Gross-Steinmeyer, K., Stapelton, P.L., Liu, F., Tracy, J.H., Bammler T.K., Quigley S.D., Farin, F.M., Buhler, D.R., Safe, S.H., Strom, S.C., Eaton, D.L., 2004. Phytochemical-induced changes in gene expression of carcinogen-metabolizing enzymes in cultured human primary hepatocytes. Xenobiotica 34, 619-632.

Groten, J.P., Butler, W.H., Feron, V.J., Kozianowski, G., Renwick, A.G., Walker, R., 2000. An analysis of the possibility for health implications of joint actions and interactions between food additives. Regul. Toxciol. Pharmacol. 31, 77-91.

Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases. Meths. Enzymol. 77, 398-405.

Habig, W.H., Prabst, M.J., Jakoby, W.H., 1974. Glutathione *S*-transferases. The first step in mercapturic acid formation. J. Biol. Chem. 249, 7130-7139.

Hegde, P., Qi, R., Abernathy, K., Gay, C., Dharap, S., Gaspard, R., Hughes, J. E., Snesrud, E., Lee, N., and Quackenbush, J. (2000). A concise guide to cDNA microarray analysis. Biotechniques 29(3), 548-4, 556.

Heijne, W. H., Stierum, R. H., Slijper, M., van Bladeren, P. J., and van Ommen, B. (2003). Toxicogenomics of bromobenzene hepatotoxicity: a combined transcriptomics and proteomics approach. Biochem. Pharmacol. 65(5), 857-875.

Hewitt, N.J., Gómez Lechon, M.J., Houston, J.B., Hallifax, D., Brown, H.S., Maurel, P., Kenna, J.G., Gustavsson, L., Lohmann, C., Skonberg, C., Guillouzo, A., Tuschl, G.,

Li, A.P., LeCluyse, E., Groothuis, G.M.M., Hengstler, J.G., 2007. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Met. Rev. 39, 159-234.

Holsapple, M.P., Pitot, H.C., Cohen, S.H., Boobis, A.R., Klaunig, J.E., Pastoor, T., Dellarco, V.L., Dragan, Y.P., 2006. Mode of action in relevance of rodent liver tumors to human cancer risk. Toxicol. Sci. 89, 51-56.

Honkakoski, P., Negishi, M., 2000. Regulation of cytochrome P450 *(CYP)* genes by nuclear receptors. Biochem. J. 347, 321-337.

Iqbal, M., Sharma, S.D., Okazaki, Y., Fujisawa, M., Okada, S., 2003. Dietary supplementation of curcumin enhances antioxidant and phase II metabolizing enzymes in ddY male mice: possible role in protection against chemical carcinogenesis and toxicity. Pharamcol. Toxciol. 92, 33-38.

Japenga, A.C., Davies, S., Price, R.J., Lake, B.G., 1993. Effect of treatment with pyrazine and some derivatives on cytochrome P450 and some enzyme activities in rat liver. Xenobiotica 23, 169-179.

Kastan, M. B., Zhan, Q., el Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71(4), 587-597.

Kikuchi, H., Kato, H., Mizuno, M., Hossain, A., Ikawa, S., Miyazaki, J., Watanabe, M., 1996. Differences in inducibility of CYP1A1-mRNA by benzimidazole compounds between human and mouse cells : evidences of a human-specific signal transduction pathway for *CYP1A1* induction. Arch. Biochem. Biophys. 334, 235-240.

Klaassen, C. D. (2001). Casarett & Doull's Toxicology: The Basic Science of Poisons, p. 193. McGraw-Hill.

Klaunig, J.E., Babich, M.A., Baetcke, K.P., Cook, J.C., Corton, J.C., David, R.M., DeLuca, J.G., Lai, D.Y., McKee, R.H., Peters, J.M., Roberts, R.A., Fenner-Crisp, P.A., 2003. PPARa agonist-induced rodent tumors: modes of action and human relevance. Crit. Rev. Toxicol. 33, 655-780.

Lake B.G., 1987. Preparation and characterisation of microsomal fractions for studies on xenobiotic metabolism, in: Snell, K., Mullock, B. (Eds.), Biochemical Toxicology: A Practical Approach. IRL Press, Oxford, pp.183-215.

Lake B.G., 1995. Mechanisms of hepatocarcinogenicity of peroxisome-proliferating drugs and chemicals. Ann. Rev. Pharmacol. Toxicol. 35, 483-507.

Lake, B. G., and Lewis, D. F. V. (1996) In Cytochromes P450 : Metabolic and Toxicological Aspects, C. Ioannides (ed), pp. 271-297, CRC Press, Boca Raton. 1996.

Lake, B.G., Renwick, A.B., Cunninghame, M.E., Price, R.J., Surry, D., Evans, D.C., 1998. Comparison of the effects of some CYP3A and other enzyme inducers on replicative DNA synthesis and cytochrome P450 isoforms in rat liver. Toxicology 131, 9-20.

Lawrence, R.A., Burk, R.F., 1976. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem. Biophys. Res. Commun. 71, 952-958.

Loewe, S., 1953. The problem of synergism and antagonism of combined drugs. Arzneimittelforschung 3, 285-290.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randal, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Madan, A., Graham, R.A., Carroll, K.M., Mudra, D.R., Burton, L.A., Krueger, L.A., Downey, A.D., Czerwinski, M., Forster, J., Ribadeneira, M.D., Gan, L.-S., LeCluyse, E.L., Zech, K., Robertson, P., Jr., Koch, P., Antonian, L., Wagner, G., Yu, L., Parkinson, A., 2003. Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. Drug Metab. Dispos. 31, 421-431.

Mannervik, B, Ålin, P., Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M, Jörnvall, H., 1985. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. Proc. Soc. Acad. Sci. USA 82, 7202-7206.

Manson, M. M., Ball, H. W., Barrett, M. C., Clark, H. L., Judah, D. J., Williamson, G., and Neal, G. E. (1997a). Mechanism of action of dietary chemoprotective agents in rat liver: induction of phase I and II drug metabolizing enzymes and aflatoxin B1 metabolism. Carcinogenesis 18(9), 1729-1738.

Manson, M. M., Ball, H. W., Barrett, M. C., Clark, H. L., Judah, D. J., Williamson, G., and Neal, G. E. (1997b). Mechanism of action of dietary chemoprotective agents in rat liver: induction of phase I and II drug metabolizing enzymes and aflatoxin B1 metabolism. Carcinogenesis 18(9), 1729-1738.

Maurel, P., 1996. The use of adult human hepatocytes in primary culture and other in vitro systems to investigate drug metabolism in man. Adv. Drug Del.Rev. 22, 105-132.

McFarlane, M., Price, S.C., Cottrell, S., Grasso, P., Bremmer, J.N., Bomhard, E.M., Hinton, R.H., 1997. Hepatic and associated response of rats to pregnancy, lactation and simultaneous treatment with butylated hydroxytoluene. Food Chem.. Toxicol. 35, 753-767.

Meredith, C., Scott, M.P., Renwick, A.B., Price, R.J., Lake B.G., 2003. Studies on the induction of rat hepatic CYP1A, CYP2B, CYP3A and CYP4A subfamily form mRNAs *in vivo* and *in vitro* using precision-cut rat liver slices. Xenobiotica 33, 511-527.

Moore, J.T., Moore, L.B., Maglich, J.M., Kliewer, S.A., 2003. Functional and structural comparison of PXR and CAR. Biochim. Biophys. Acta 1619, 235-238.

Mori, Y., Tatematsu, K., Koide, A., Sugie, S., Tanaka, T., Mori, H., 2006. Modification by curcumin of mutagenic activation of carcinogenic *N*-nitrosamines by extrahepatic cytochromes P-450 2B1 and 2E1 in rats. Cancer Sci. 97, 896-904.

Nerurkar, P.V., Park, S.S., Thomas, P.E., Nims, R.W., Lubet, R.A., 1993. Methoxyresorufin and benzyloxyresorufin : substrates preferentially metabolized by cytochromes P4501A2 and 2B, respectively, in the rat and mouse. Biochem. Pharmacol. 46, 933-943.

Nims, R.W., Lubet, R.A., 1996. The CYP2B subfamily, in: Ioannides, C. (Ed.), Cytochromes P450 : Metabolic and Toxicological Aspects. CRC Press, Boca Raton, pp. 135-160.

Okey, A.B., 1990. Enzyme induction in the cytochrome P-450 system. Pharmacol. Ther. 45, 241-298.

Oetari, S., Sudibyo, M., Commandeur, J.N., Samhoedi, R., Vermeulen, N.P., 1996. Effects of curcumin on cytochrome P450 and glutathione S-transferase activities in rat liver. Biochem. Pharmacol. 51, 39-45.

Parkinson, A., 2001. Biotransformation of xenobiotics, in: Klaassen, C.D. (Ed.), Casarett and Doull's Toxicology : The Basic Science of Poisons, 6th ed. McGraw Hill, New York, pp. 133–224.

Parkinson, A., Mudra, D.R., Johnson, C., Dwyer, A., Carroll, K.M., 2004. The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome *P*450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. Toxicol. Appl. Pharmacol. 199, 193-209.

Pelkonen, O., Mäenpää, J., Taavitsainen, P., Rautio, A., Raunio, H., 1998. Inhibition and induction of human cytochrome P450 (CYP) enzymes. Xenobiotica 28, 1203-1253.

Price, R.J., Giddings, A.M., Scott, M.P., Walters, D.G., Capen, C., Osimitz, T.G., Lake, B.G., 2008. Effect of Pyrethrins on cytochrome P450 forms in cultured rat and human hepatocytes. Toxicology 243, 85-95.

Price, R.J., Scott, M.P., Walters, D.G., Stierum, R.H., Groten, J.P., Meredith, C., Lake, B.G., 2004. Effect of thiabendazole on some rat hepatic xenobiotic metabolising enzymes. Food Chem. Toxicol. 42, 899-908.

Price, R.J., Surry, D., Renwick, A.B., Meneses-Lorente, G., Lake, B.G., Evans, D.C., 2000. CYP isoform induction screening in 96-well plates: use of 7-benzyloxy-4-trifluoromethylcoumarin as a substrate for studies with rat hepatocytes. Xenobiotica 30, 781-795.

Raucy, J.L., 2003. Regulation of CYP3A4 expression in human hepactoytes by pharmaceuticals and natural products. Drug Metab. Dispos. 31, 533-539.

Rey-Grobellet, X., Ferre, N., Eeckhoutte, C., Larrieu, G., Pineau, T., Galtier, P., 1996. Structural requirements for the induction of cytochromes P450 by benzimidazole anthelmintic derivatives in cultured rabbit hepatocytes. Biochem. Biophys. Res. Commun. 220, 789-794.

Rinaldi, A. L., Morse, M. A., Fields, H. W., Rothas, D. A., Pei, P., Rodrigo, K. A., Renner, R. J., and Mallery, S. R. (2002). Curcumin activates the aryl hydrocarbon receptor yet significantly inhibits (-)-benzo(a)pyrene-7R-trans-7,8-dihydrodiol bioactivation in oral squamous cell carcinoma cells and oral mucosa. Cancer Res. 62(19), 5451-5456.

Scivittaro, V., Ganz, M. B., and Weiss, M. F. (2000). AGEs induce oxidative stress and activate protein kinase C-beta(II) in neonatal mesangial cells. Am. J. Physiol Renal Physiol 278(4), F676-F683.

Sharma, R.A., Gescher, A.J., Steward, W.P., 2005. Curcumin: the story so far. Eur. J. Cancer 41, 1955-1968.

Sharma, R.A., Ireson, C.R., Verschoyle, R.D., Hill, K.A., Williams, M.L., Leuratti, C., Manson, M.M., Marnett, L.J., Steward, W.P., Gescher, A., (2001). Effects of dietary curcumin on glutathione *S*-transferase and malondialdehyde-DNA adducts in rat liver and colon mucosa: relationship with drug levels. Clin. Cancer Res. 7, 1452-1458.

Sherratt P.J., Hayes, J.D., 2002. Glutathione *S*-transferases, in: Ioannides, C. (Ed.), Enzyme Systems that Metabolise Drugs and Other Xenobiotics. John Wiley, Chichester, pp. 319-352.

Singh, S.V., Hu, X., Srivastava, S.K., Singh, M., Xia, H., Orchard, J.L., Zaren, H.A., 1998. Mechanism of inhibition of benzo[*a*]pyrene-induced forestomach cancer in mice by dietary curcumin. Carcinogenesis 19, 1357-1360.

Stewart, J., Boston, C.M., 1987. Effect of pretreatment with 3-methylcholanthrene and several phenolic antioxidants on glutathione S-transferase activity in various tissues of female Sprague-Dawley rats. Xenobiotica 17, 679-684.

Stewart, J., McCrary, J.P., 1987. Comparison of the glucuronidation ability of liver microsomes from rats treated with 3-methylcholanthrene or phenolic antioxidants. Xenobiotica 17, 1039-1046.

Stierum, R. H., Conesa, A., Heijne, W., van Ommen, B., Junker K., Scott, M. P., Price, R. J., Meredith, C., Lake, B. G., and Groten, J. P. (2008). Transcriptome analysis provides new insights into liver changes induced in the rat upon dietary administration of the food additives butylated hydroxytoluene, curcumin, propyl gallate and thiabendazole. Food Chem. Toxicol. 46, 2616-2628.

Stubberfield, C.R., Cohen, G.M., 1989. Interconversion of NAD(H) to NADP(H). A cellular response to quinone-induced oxidative stress in isolated hepatocytes. Biochem. Pharmacol. 38, 2631-2637.

Sun, B., Fukuhara, M., 1997. Effects of co-administration of butylated hydroxytoluene, butylated hydroxyanisole and flavonoids on the activities of mutagens and drug-metabolizing enzymes in mice. Toxicology 122, 61-72.

Sun, B., Fukuhara, M., Kinoshita, T., Kimura, M., Ushio, F., 1996. Differential induction of drug-metabolizing enzymes by butylated hydroxytoluene in mice and Chinese hamsters. Food Chem. Toxciol. 34, 595-601.

Suzan, M., Rao, M.N.A., 1992. Induction of glutathione S-transferase activity in mice. Arzn.-Forsch/Drug res. 42, 962-964.

Thapliyal, R., Maru, G.B., 2001. Inhibition of cytochrome P450 isozymes by curcumin in vitro and in vivo. Food Chem. Toxciol. 39, 541-547.

Walton, K., Walker, R., van de Sandt, J.J.M., Castell, J.V., Knapp, A.G.A.A., Kozianowski, G., Roberfroid, M., Schilter, B, 1999. The application of *in vitro* data in the derivation of the acceptable daily intake of food additives. Food Chem. Toxciol. 37, 1175-1197.

Wang, H., Faucette, S.R., Gilbert, D., Jolley, S.L., Sueyoshi, T., Negishi, M., LeCluyse, E.L., 2003. Glucocorticoid receptor enhancement of pregnane X receptor-mediated CYP2B6 regulation in primary human hepatocytes. Drug Metab. Dispos. 31, 620-630.

Wu, L. X., Xu, J. H., Huang, X. W., Zhang, K. Z., Wen, C. X., and Chen, Y. Z. (2006). Down-regulation of p210(bcr/abl) by curcumin involves disrupting molecular chaperone functions of Hsp90. Acta Pharmacol. Sin. 27(6), 694-699.

Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., and Speed, T. P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. 30(4), e15.

Zhan, Q., Bae, I., Kastan, M. B., and Fornace, A. J., Jr. (1994). The p53-dependent gamma-ray response of GADD45. Cancer Res. 54(10), 2755-2760.

Zimmerman, H.J., 1978. The Adverse Effects of Drugs and Other Chemicals on the Liver. Appleton-Century-Crofts, New York, p. 342.

10. TABLES

Table 3. Calculated and actual dietary levels of BHT, CC, PG and TB

Group	Compound	Target compound concentration (ppm in diet)[a]	Actual compound concentration (ppm in diet)[b]	Percentage of target daily intake
A	Control	–	–	–
B	BHT	254	239.5	94
C	BHT	762	743.9	98
D	BHT	1523	1456.6	96
E	BHT	3046	2859.9	94
F	BHT	10154	10155.2	100
G	CC	254	239.9	95
H	CC	762	739.6	97
I	CC	2031	2033.6	100
J	CC	4062	4164.8	103
K	CC	10154	10333.0	102
L	PG	203	184.6	91
M	PG	508	453.1	89
N	PG	1523	1463.7	96
O	PG	3046	3279.9	108
P	PG	6092	6124.0	101
Q	TB	102	102.3	100
R	TB	254	248.9	98
S	TB	762	757.5	99
T	TB	2031	2016.2	99
U	TB	5077	5188.4	102

[a]Calculated from desired mg/kg/day intake and body weight and food consumption data.

[b]Analysed levels of BHT, CC, PG and TB in study diets.

Table 4. Effect of treatment of rats for 3 days with BHT, CC, PG and TB on body weight, liver weight and relative liver weight

Group	Treatment (ppm)[a]	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
A	Control	169.9 ± 4.0 (100)[b]	8.1 ± 0.5 (100)	4.74 ± 0.24 (100)
D	BHT 1523	168.8 ± 7.7 (99)	9.4 ± 0.6 (116)**	5.54 ± 0.24 (117)***
F	BHT 10154	149.0 ± 4.7 (88)***	10.6 ± 1.1 (131)***	7.08 ± 0.63 (149)***
I	CC 2031	167.7 ± 9.2 (99)	8.1 ± 0.5 (100)	4.81 ± 0.22 (101)
K	CC 10154	166.2 ± 7.4 (98)	7.6 ± 0.3 (94)	4.58 ± 0.11 (97)
N	PG 1523	159.2 ± 4.5 (94)**	7.1 ± 0.5 (88)*	4.46 ± 0.26 (94)
P	PG 6092	156.5 ± 9.1 (92)**	7.3 ± 0.7 (91)	4.67 ± 0.33 (99)
S	TB 762	161.8 ± 10.9 (95)*	8.2 ± 1.1 (101)	5.03 ± 0.45 (106)
U	TB 5077	133.2 ± 10.0 (78)***	6.3 ± 1.1 (78)***	4.69 ± 0.56 (99)

[a]Dietary levels are target levels (ppm in diets).

[b]Results are presented as mean ± SD for groups of 10 control (group A) and 6 treated (all other groups) rats. Percentage of control values are shown in parentheses.

Values significantly different from control are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 6. Effect of treatment of rats for 28 days with BHT, CC, PG and TB on serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activity

Group	Treatment (ppm)[a]	Serum enzyme activity (U/l)[b]		
		ALT	AST	LDH
A	Control	72 ± 11 (100)[c]	79 ± 11 (100)	120 ± 33 (100)
B	BHT 254	65 ± 5 (90)	78 ± 6 (99)	137 ± 12 (114)
C	BHT 762	68 ± 13 (94)	73 ± 5 (92)	150 ± 89 (125)
D	BHT 1523	63 ± 8 (88)	73 ± 8 (92)	138 ± 25 (115)
E	BHT 3046	60 ± 4 (83)**	71 ± 5 (90)	234 ± 240 (195)*
F	BHT 10154	72 ± 5 (100)	76 ± 6 (96)	130 ± 44 (108)
G	CC 254	82 ± 9 (114)	80 ± 6 (101)	119 ± 8 (99)
H	CC 762	80 ± 9 (111)	82 ± 7 (104)	131 ± 26 (109)
I	CC 2031	72 ± 18 (100)	76 ± 12 (96)	123 ± 16 (103)
J	CC 4062	72 ± 11 (100)	75 ± 6 (95)	99 ± 10 (83)
K	CC 10154	80 ± 11 (111)	94 ± 30 (119)*	129 ± 11 (108)
L	PG 203	76 ± 11 (106)	77 ± 3 (98)	134 ± 44 (112)
M[d]	PG 508	76 ± 3 (106)	74 ± 4 (94)	105 ± 5 (88)
N	PG 1523	73 ± 11 (101)	80 ± 8 (101)	132 ± 40 (110)
O	PG 3046	77 ± 7 (107)	82 ± 6 (104)	117 ± 19 (98)
P	PG 6092	73 ± 13 (101)	77 ± 6 (98)	148 ± 53 (123)
Q	TB 102	74 ± 13 (103)	78 ± 11 (99)	128 ± 26 (107)
R	TB 254	74 ± 16 (103)	78 ± 13 (99)	102 ± 17 (85)
S	TB 762	71 ± 5 (99)	77 ± 3 (98)	118 ± 17 (98)
T	TB 2031	98 ± 21 (136)***	80 ± 11 (101)	127 ± 21 (106)
U	TB 5077	79 ± 10 (110)	73 ± 6 (92)	162 ± 31 (135)**

[a]Dietary levels are target levels (ppm in diets).

[b]Assays performed at BIBRA.

[c]Results are presented as mean ± SD for groups of 10 control (group A) and either 5 (groups M and U only) or 6 (all other groups) treated rats. Percentage of control values are shown in parentheses.

[d]One serum sample from this group was haemolysed and hence the data for this animal was excluded from the analysis.

Values significantly different from control are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 7. Effect of treatment of rats for 28 days with BHT, CC, PG and TB on serum sorbital dehydrogenase (SDH), total protein, albumin, triglycerides and cholesterol

Group	Treatment (ppm)[a]	Serum parameter[b]				
		SDH (U/l)	Total protein (g/l)	Albumin (g/l)	Triglycerides (mmol/l	Cholesterol (mmol/l)
A	Control	13.4 ± 4.3 (100)[c]	65 ± 2.5 (100)	42 ± 1.7 (100)	1.09 ± 0.28 (100)	3.02 ± 0.31 (100)
B	BHT 254	13.9 ± 2.3 (104)	65 ± 3.1 (100)	42 ± 1.7 (100)	1.04 ± 0.27 (95)	2.89 ± 0.12 (96)
C	BHT 762	14.6 ± 3.5 (109)	67 ± 2.0 (103)	43 ± 0.8 (102)	1.15 ± 0.33 (106)	3.19 ± 0.23 (106)
D	BHT 1523	15.5 ± 3.6 (116)	68 ± 2.6 (105)	43 ± 1.3 (102)	1.08 ± 0.26 (99)	3.24 ± 0.67 (107)
E	BHT 3046	14.4 ± 1.9 (108)	67 ± 1.9 (103)	42 ± 1.2 (100)	0.93 ± 0.24 (85)	3.54 ± 0.31 (117)*
F	BHT 10154	19.4 ± 3.5 (145)*	68 ± 3.1 (105)	42 ± 1.9 (100)	0.75 ± 0.22 (69)*	4.33 ± 0.42 (143)***
G	CC 254	14.0 ± 1.9 (105)	66 ± 1.9 (102)	43 ± 1.0 (102)	0.95 ± 0.12 (87)	2.83 ± 0.26 (94)
H	CC 762	18.5 ± 2.8 (138)**	66 ± 3.3 (102)	43 ± 1.4 (102)	1.01 ± 0.15 (93)	2.99 ± 0.17 (99)
I	CC 2031	16.9 ± 3.8 (126)*	64 ± 3.2 (99)	42 ± 2.0 (100)	0.92 ± 0.41 (84)	2.66 ± 0.42 (88)*
J	CC 4062	14.0 ± 2.6 (105)	65 ± 2.6 (100)	44 ±1.2 (105)	0.70 ± 0.21 (64)**	2.52 ± 0.14 (83)**
K	CC 10154	14.1 ±2.4 (105)	67 ± 3.3 (103)	44 ± 2.4 (105)	0.73 ± 0.12 (67)**	2.72 ± 0.25 (90)*
L	PG 203	15.1 ± 2.4 (113)	68 ± 4.3 (105)	43 ± 1.7 (102)	0.91 ± 0.26 (84)	2.94 ± 0.52 (97)
M[d]	PG 508	13.1 ± 2.4 (98)	66 ± 1.6 (102)	43 ± 0.8 (102)	0.85 ± 0.25 (78)	2.89 ± 0.15 (96)
N	PG 1523	15.7 ± 4.0 (117)	65 ± 4.9 (100)	42 ± 2.8 (100)	1.12 ± 0.24 (103)	2.56 ± 0.34 (85)*
O	PG 3046	14.2 ± 2.4 (106)	67 ± 2.2 (103)	44 ± 1.6 (105)	1.08 ± 0.38 (99)	2.71 ± 0.25 (90)
P	PG 6092	17.8 ± 4.2 (133)*	65 ± 2.4 (100)	43 ± 1.3 (102)	1.51 ± 0.32 (139)**	2.56 ± 0.47 (85)*
Q	TB 102	15.3 ± 5.0 (114)	62 ± 5.9 (95)	41 ± 3.1 (98)	1.00 ± 0.23 (92)	3.01 ± 0.49 (100)
R	TB 254	12.9 ± 3.6 (96)	66 ± 2.0 (102)	43 ± 2.3 (102)	0.89 ± 0.43 (82)	2.82 ± 0.50 (93)
S	TB 762	13.2 ± 1.9 (99)	66 ± 2.9 (102)	43 ± 1.6 (102)	1.05 ± 0.30 (96)	3.08 ± 0.53 (102)
T	TB 2031	18.7 ± 3.8 (140)*	70 ± 2.9 (108)**	47 ± 1.4 (112)***	0.86 ± 0.18 (79)	3.96 ± 0.52 (131)***
U	TB 5077	17.8 ± 3.1 (133)*	76 ± 3.3 (117)***	49 ± 1.4 (117)***	1.02 ± 0.34 (94)	4.74 ± 0.76 (157)***

[a]Dietary levels are target levels (ppm in diets).
[b]Assays performed at TNO.
[c]Results are presented as mean ± SD for groups of 10 control (group A) and either 5 (groups M and U only) or 6 (all other groups) treated rats. Percentage of control values are shown in parentheses.
[d]One serum sample from this group was haemolysed and hence the data for this animal was excluded from the analysis.

Values significantly different from control are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 8. Effect of treatment of rats for 28 days with BHT, CC, PG and TB on hepatic microsomal and cytosolic protein content

Group	Treatment (ppm)[a]	Microsomal protein (mg/g liver)	Cytosolic protein (mg/g liver)
A	Control	37.2 ± 1.1 (100)[b]	94.9 ± 1.5 (100)
B	BHT 254	37.7 ± 1.1 (102)	95.0 ± 2.2 (100)
C	BHT 762	38.1 ± 1.7 (102)	89.2 ± 2.4 (94)*
D	BHT 1523	38.9 ± 0.8 (105)	89.4 ± 2.1 (94)*
E	BHT 3046	41.4 ± 0.8 (111)**	85.4 ± 1.3 (90)***
F	BHT 10154	43.1 ± 0.8 (116)***	83.7 ± 1.6 (88)***
G	CC 254	41.1 ± 1.6 (111)*	117.7 ± 6.5 (124)***
H	CC 762	39.5 ± 0.8 (106)	94.9 ± 3.3 (100)
I	CC 2031	36.4 ± 1.2 (98)	95.0 ± 2.5 (100)
J	CC 4062	37.7 ± 0.8 (101)	99.7 ± 3.4 (105)
K	CC 10154	37.2 ± 0.8 (100)	93.4 ± 2.4 (98)
L	PG 203	36.7 ± 2.1 (99)	100.6 ± 2.1 (106)
M	PG 508	34.3 ± 1.1 (92)	99.1 ± 2.4 (98)
N	PG 1523	33.4 ± 2.0 (90)	92.7 ± 4.1 (98)
O	PG 3046	41.1 ± 0.9 (111)*	125.7 ± 8.7 (132)***
P	PG 6092	40.7 ± 1.1 (110)	145.7 ± 0.3 (154)***
Q	TB 102	37.0 ± 2.1 (100)	92.3 ± 1.8 (97)
R	TB 254	35.9 ± 1.2 (97)	93.8 ± 2.5 (99)
S	TB 762	37.9 ± 0.9 (102)	94.0 ± 2.5 (99)
T	TB 2031	38.5 ± 0.6 (104)	99.2 ± 3.9 (104)
U	TB 5077	40.3 ± 1.2 (108)	109.0 ± 2.1 (115)***

[a]Dietary levels are target levels (ppm in diets).

[b]Results are presented as mean ± SEM for groups of 10 control (group A) and 6 treated (groups B to U) rats. Percentage of control values are shown in parentheses.

Values significantly different from control are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 10. Effect of treatment of rats for 28 days with BHT, CC, PG and TB on hepatic CYP2B1 and CYP2B1/2 mRNA levels

Group	Treatment (ppm)[a]	CYP mRNA level (fold induction)[b]	
		CYP2B1	CYP2B1/2
A	Control	1.00 ± 0.06 (100)[c]	1.00 ± 0.05
B	BHT 254	6.96 ± 1.69***	8.28 ± 2.07***
C	BHT 762	84.93 ± 24.08***	65.15 ± 9.25***
D	BHT 1523	442.0 ± 120.9***	99.13 ± 16.92***
E	BHT 3046	736.8 ± 129.7***	355.2 ± 23.2***
F	BHT 10154	3114.8 ± 646.2***	971.7 ± 169.2***
G	CC 254	2.98 ± 0.36*	1.27 ± 0.21
H	CC 762	11.57 ± 4.25***	0.70 ± 0.13
I	CC 2031	11.14 ± 4.45***	1.88 ± 0.36
J	CC 4062	6.09 ± 3.29**	2.01 ± 0.54*
K	CC 10154	6.71 ± 2.60**	3.34 ± 0.61***
L	PG 203	2.31 ± 1.10	0.40 ± 0.08***
M	PG 508	4.44 ± 2.08	0.89 ± 0.18
N	PG 1523	3.41 ± 1.88	0.39 ± 0.11***
O	PG 3046	0.43 ± 0.07	0.48 ± 0.10**
P	PG 6092	8.85 ± 6.15*	0.71 ± 0.18
Q	TB 102	0.40 ± 0.15**	0.25 ± 0.06***
R	TB 254	1.37 ± 0.57	0.50 ± 0.23**
S	TB 762	0.59 ± 0.19*	0.57 ± 0.20
T	TB 2031	5.04 ± 2.01**	5.26 ± 1.00***
U	TB 5077	40.46 ± 11.86***	24.59 ± 4.79***

[a]Dietary levels are target levels (ppm in diets).

[b]To normalise for RNA loading, levels of CYP mRNAs were expressed as a ratio to levels of albumin mRNA which was co-amplified in a duplex reaction.

[c]Results are presented as mean ± SEM for groups of 10 control (group A) and 6 treated (groups B to U) rats.

Values significantly different from control are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 12 Effect of treatment of rats for 28 days with BHT, CC, PG and TB on hepatic cytosolic GST activities towards TPBO, EA and ENPP as substrates

Group	Treatment (ppm)[a]	GST activity (μmol/min/g liver)[b]		
		Substrate TPBO	Substrate EA	Substrate ENPP
A	Control	1.55 ± 0.08 (100)[c]	2.21 ± 0.07 (100)	8.58 ± 0.32 (100)
B	BHT 254	n.d.[d]	2.31 ± 0.07 (104)	8.37 ± 0.44 (98)
C	BHT 762	n.d.	2.54 ± 0.10 (115)	9.12 ± 0.39 (106)
D	BHT 1523	n.d.	2.68 ± 0.07 (121)*	11.98 ± 0.58 (140)***
E	BHT 3046	n.d.	3.16 ± 0.15 (143)***	11.95 ± 0.72 (139)***
F	BHT 10154	n.d.	5.32 ± 0.37 (241)***	14.01 ± 0.76 (163)***
G	CC 254	1.61 ± 0.05 (104)	2.49 ± 0.11 (113)	8.64 ± 0.22 (101)
H	CC 762	1.58 ± 0.04 (102)	2.42 ± 0.11 (110)	7.87 ± 0.39 (92)
I	CC 2031	1.56 ± 0.13 (101)	2.45 ± 0.09 (111)	8.26 ± 0.50 (96)
J	CC 4062	1.84 ± 0.06 (119)*	2.54 ± 0.09 (115)*	8.12 ± 0.33 (95)
K	CC 10154	1.73 ± 0.10 (112)	2.50 ± 0.08 (113)*	6.82 ± 0.41 (79)**
L	PG 203	1.45 ± 0.07 (93)	2.14 ± 0.12 (97)	8.97 ± 0.24 (105)
M	PG 508	1.70 ± 0.07 (110)	2.11 ± 0.13 (96)	8.52 ± 0.61 (99)
N	PG 1523	1.72 ± 0.14 (111)	2.11 ± 0.10 (95)	9.40 ± 0.45 (110)
O	PG 3046	1.80 ± 0.20 (116)	2.50 ± 0.12 (113)	8.95 ± 0.43 (104)
P	PG 6092	1.85 ± 0.18 (120)	2.29 ± 0.11 (104)	10.66 ± 0.95 (124)**
Q	TB 102	n.d.	2.46 ± 0.14 (111)	9.15 ± 0.26 (107)
R	TB 254	n.d.	2.60 ± 0.06 (118)	9.98 ± 0.32 (116)*
S	TB 762	n.d.	2.81 ± 0.20 (127)**	10.75 ± 0.63 (125)***
T	TB 2031	n.d.	4.24 ± 0.22 (192)***	13.91 ± 0.41 (162)***
U	TB 5077	n.d.	6.89 ± 0.29 (312)***	14.22 ± 0.62 (166)***

[a]Dietary levels are target levels (ppm in diets).

[b]GST substrates were: TPBO, *trans*-4-phenyl-3-buten-2-one; EA, ethacrynic acid; ENPP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane.

[c]Results are presented as mean ± SEM for groups of 10 control (group A) and 6 treated (groups B to U) rats. Percentage of control values are shown in parentheses.

[d]n.d.; not determined.

Values significantly different from control are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 13. Effect of treatment of rats for 28 days with BHT, CC, PG and TB on hepatic GSTP1 and GSTT1 mRNA levels

Group	Treatment (ppm)[a]	GST mRNA level (fold induction)[b,c]			
		GSTP1		GSTT1	
A	Control	1.00	± 0.04	1.00	± 0.03
B	BHT 254	1.99	± 0.30	1.05	± 0.09
C	BHT 762	1.95	± 0.65	1.25	± 0.31
D	BHT 1523	1.45	± 0.24	1.71	± 0.15*
E	BHT 3046	2.02	± 0.53	1.71	± 0.25*
F	BHT 10154	102.07	± 35.57***	4.05	± 0.54***
G	CC 254	9.68	± 6.59*	1.92	± 0.34**
H	CC 762	34.14	± 22.89***	1.52	± 0.22
I	CC 2031	29.79	± 21.64***	1.15	± 0.17
J	CC 4062	4.32	± 3.49	0.88	± 0.10
K	CC 10154	4.84	± 2.37	0.86	± 0.14
L	PG 203	1.48	± 0.34	1.05	± 0.12
M	PG 508	1.33	± 0.48	1.67	± 0.14*
N	PG 1523	3.99	± 0.88*	1.75	± 0.27*
O	PG 3046	15.24	± 12.52*	1.49	± 0.28
P	PG 6092	22.27	± 12.69**	1.61	± 0.19
Q	TB 102	1.32	± 0.43	1.09	± 0.19
R	TB 254	4.01	± 1.62	1.31	± 0.27
S	TB 762	3.61	± 0.87	1.77	± 0.33*
T	TB 2031	28.92	± 5.38***	2.67	± 0.43***
U	TB 5077	248.22	± 58.47***	3.22	± 0.43***

[a]Dietary levels are target levels (ppm in diets).
[b]To normalise for RNA loading, levels of GST mRNAs were expressed as a ratio to levels of albumin mRNA which was co-amplified in a duplex reaction.
[c]Results are presented as mean ± SEM for groups of 10 control (group A) and 6 treated (groups B to U) rats.

Values significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

Table 15a. Design of rat *in vivo* mixtures study with BHT, CC, PG and TB

Group	Compound dose level in diet[a]			
	Compound 1	Compound 2	Compound 3	Compound 4
1	0	0	0	0
2	25	0	0	0
3	50	0	0	0
4	100	0	0	0
5	0	25	0	0
6	0	50	0	0
7	0	100	0	0
8	0	0	25	0
9	0	0	50	0
10	0	0	100	0
11	0	0	0	25
12	0	0	0	50
13	0	0	0	100
14	50	50	0	0
15	50	0	50	0
16	50	0	0	50
17	0	50	50	0
18	0	50	0	50
19	0	0	50	50
20	25	25	25	25
21	25	25	0	0
22	25	0	25	0
23	25	0	0	25
24	0	25	25	0
25	0	25	0	25
26	0	0	25	25
27	12.5	12.5	12.5	12.5
28	6.25	6.25	6.25	6.25

[a]The highest dose level of each compound is defined as 100 units.
Group 1 is the control group.
Groups 2 to 13 are individual compound dose levels (25, 50 and 100 units).
Groups 14 to 19 are binary mixture combinations comprising 50 units of each food additive.
Groups 19 to 24 are binary mixture combinations comprising 25 units of each food additive.
Groups 20, 27 and 28 are quaternary mixtures comprising 25, 12.5 and 6.25 units, respectively, of each food additive.

Table 15b. Design in vivo mixture study: fractional, nominal and actual dose levels of BHT, CC, PG and TB employed in binary and quaternary mixtures

Group	BHTf[1,6]	BHTn[2]	BHTa[3]	CCf	CCn	CCa	PGf	PGn	PGa	TBf	TBn	TBa
1: Control	0	0	0	0	0	0	0	0	0	0	0	0
2: BHT25[4]	25	762	752	0	0	0	0	0	0	0	0	0
3: BHT50	50	1523	1574	0	0	0	0	0	0	0	0	0
4: BHT100	100	3046	3289	0	0	0	0	0	0	0	0	0
5: CC25	0	0	0	25	1016	103 0	0	0	0	0	0	0
6: CC50	0	0	0	50	2031	211 2	0	0	0	0	0	0
7: CC100	0	0	0	100	4062	430 6	0	0	0	0	0	0
8: PG25	0	0	0	0	0	0	25	762	813	0	0	0
9: PG50	0	0	0	0	0	0	50	152 3	167 7	0	0	0
10: PG100	0	0	0	0	0	0	100	304 6	314 2	0	0	0
11: TB25	0	0	0	0	0	0	0	0	0	25	438	456
12: TB50	0	0	0	0	0	0	0	0	0	50	875	920
13: TB100	0	0	0	0	0	0	0	0	0	100	175 0	184 2
14: BHT_CC100	50	1523	1492	50	2031	212 5	0	0	0	0	0	0
15: BHT_PG100	50	1523	1551	0	0	0	50	152 3	163 2	0	0	0
16: BHT_TB100	50	1523	1523	0	0	0	0	0	0	50	875	924
17: CC_PG100	0	0	0	50	2031	208 4	50	152 3	157 4	0	0	0
18: CC_TB100	0	0	0	50	2031	206 2	0	0	0	50	875	910
19: PG_TB100	0	0	0	0	0	0	50	152 3	156 8	50	875	906
20: all_100	25	762	698	25	1016	994 100	25	762	732	25	438	444
21: BHT_CC50	25	762	762	25	1016	0	0	0	0	0	0	0
22: BHT_PG50	25	762	799	0	0	0	25	762	720	0	0	0
23: BHT_TB50	25	762	774	0	0	0	0	0	0	25	438	460
24: CC_PG50	0	0	0	25	1016	105 2	25	762	767	0	0	0
25: CC_TB50[5]	0	0	0	25	1016	105 0	0	0	0	25	438	457
26: PG_TB50	0	0	0	0	0	0	25	762	821	25	438	458

27: all_50	12.5	381	377	12.5	508	519	12.5	381	383	12.5	219	230
28: all_25	6.25	190	218	6.25	254	243	6.25	190	184	6.25	109	112

[1] 'f' indicates fractional dosage: the dose level expressed as percentage of the maximum dose level employed for each individual additive.

[2] 'n' indicates nominal or target dosage: the dose level intended to be administered.

[3] 'a' indicates actual dosage, the dose level actually determined in the diet.

[4] example BHT25, indicates single compound exposure, at fractional dosages of 25%, derived from BHT at nominal dosage of 762 devided by BHT at maximal nominal dosage of 3046 ppm = 0.25 (expressed as 25%).

[5] example CC_TB50, indicates binary mixture exposure, at fractional dosages of 25% of each of the individual compounds. Fractional dosage of CC in mixture: CC nominal dosage of 1015.5 ppm/CC maximal dosage of 4062 ppm = 0.25 and fractional dosage of TB in mixture: TB nominal dosage of 437.5 ppm/TB maximal dosage of 1750 ppm = 0.25 , together 50%, expressed as percentage.

[6] Actual fractional dose level employed in statistical calculations equals (actual dose level/actual maximum dose level employed) *100 (e.g. for BHT25: (752/3289)*100=23. For CC_TB50: CC (1050/4306)*100=24; TB (457/1842)*100=25).

Table 16. Analysis of study diets

Group	Animal numbers	Treatment	Target compound concentration (ppm in diet)				Actual compound concentration (ppm in diet)				Percentage of target dietary concentration			
			BHT	CC	PG	TB	BHT	CC	PG	TB	BHT	CC	PG	TB
1	1 - 12	Control	-	-	-	-	-	-	-	-	-	-	-	-
2	13 - 18	BHT	762	-	-	-	751.6	-	-	-	98.7	-	-	-
3	19 - 24	BHT	1523	-	-	-	1574.4	-	-	-	103.4	-	-	-
4	25 - 30	BHT	3046	-	-	-	3289.5	-	-	-	108.0	-	-	-
5	31 - 36	CC	-	1016	-	-	-	1030.4	-	-	-	101.5	-	-
6	37 - 42	CC	-	2031	-	-	-	2112.0	-	-	-	104.0	-	-
7	43 - 48	CC	-	4062	-	-	-	4306.0	-	-	-	106.0	-	-
8	49 - 54	PG	-	-	762	-	-	-	813.3	-	-	-	106.8	-
9	55 - 60	PG	-	-	1523	-	-	-	1677.4	-	-	-	110.1	-
10	61 - 66	PG	-	-	3046	-	-	-	3141.8	-	-	-	103.1	-
11	67 - 72	TB	-	-	-	438	-	-	-	455.6	-	-	-	104.1
12	73 - 78	TB	-	-	-	875	-	-	-	919.7	-	-	-	105.1
13	79 - 84	TB	-	-	-	1750	-	-	-	1842.2	-	-	-	105.3
14	85 - 90	BHT/CC	1523	2031	-	-	1492.2	2125.3	-	-	98.0	104.6	-	-
15	91 - 96	BHT/PG	1523	-	1523	-	1551.0	-	1632.2	-	101.8	-	107.2	-
16	97 - 102	BHT/TB	1523	-	-	875	1522.7	-	-	923.6	100.0	-	-	105.5
17	103 - 108	CC/PG	-	2031	1523	-	-	2084.1	1574.2	-	-	102.6	103.4	-
18	109 - 114	CC/TB	-	2031	-	875	-	2061.9	-	910.0	-	101.5	-	104.0
19	115 - 120	PG/TB	-	-	1523	875	-	-	1568.4	905.7	-	-	103.0	103.5
20	121 - 126	BHT/CC/PG/TB	762	1016	762	438	697.8	993.9	731.6	443.5	91.6	97.9	96.1	101.4
21	127 - 132	BHT/CC	762	1016	-	-	761.5	999.9	-	-	100.0	98.5	-	-
22	133 - 138	BHT/PG	762	-	762	-	799.2	-	720.2	-	105.0	-	94.6	-
23	139 - 144	BHT/TB	762	-	-	438	774.1	-	-	459.9	101.6	-	-	105.1
24	145 - 150	CC/PG	-	1016	762	-	-	1051.7	766.9	-	-	103.6	100.7	-
25	151 - 156	CC/TB	-	1016	-	438	-	1049.9	-	457.3	-	103.4	-	104.5
26	157 - 162	PG/TB	-	-	762	438	-	-	821.1	457.6	-	-	107.8	104.6
27	163 - 168	BHT/CC/PG/TB	381	508	381	219	377.0	519.4	383.4	229.7	99.0	102.3	100.7	105.0
28	169 - 174	BHT/CC/PG/TB	190	254	190	109	218.0	242.5	184.4	111.5	114.5	95.5	96.9	102.0

Table 17. Mean daily food consumption and calculated daily intakes for male Sprague-Dawley rats fed diets containing various combinations of BHT, CC, PB and TB for 28 days

Group	Treatment	Actual compound concentration (ppm in diet)				Mean food consumption (g/rat/day)[a]	Compound daily intake (mg/kg/day)[b]			
		BHT	CC	PG	TB		BHT	CC	PG	TB
1	Control	-	-	-	-	23.5	-	-	-	-
2	BHT	752	-	-	-	22.9	71.7	-	-	-
3	BHT	1574	-	-	-	22.3	150	-	-	-
4	BHT	3290	-	-	-	23.9	333	-	-	-
5	CC	-	1030	-	-	25.1	-	104	-	-
6	CC	-	2112	-	-	26.0	-	226	-	-
7	CC	-	4306	-	-	22.7	-	408	-	-
8	PG	-	-	813	-	22.4	-	-	76.0	-
9	PG	-	-	1677	-	22.4	-	-	156	-
10	PG	-	-	3142	-	21.6	-	-	290	-
11	TB	-	-	-	456	22.2	-	-	-	42.5
12	TB	-	-	-	920	22.1	-	-	-	85.5
13	TB	-	-	-	1842	17.9	-	-	-	153
14	BHT/CC	1492	2125	-	-	23.4	147	210	-	-
15	BHT/PG	1551	-	1632	-	21.8	145	-	153	-
16	BHT/TB	1523	-	-	924	21.4	145	-	-	87.7
17	CC/PG	-	2084	1574	-	23.8	-	204	154	-
18	CC/TB	-	2062	-	910	21.6	-	187	-	82.5
19	PG/TB	-	-	1568	906	19.7	-	-	137	79.3
20	BHT/CC/PG/TB	698	994	732	444	23.5	66.5	94.8	69.8	42.3
21	BHT/CC	762	1000	-	-	23.0	74.6	97.9	-	-
22	BHT/PG	799	-	720	-	22.7	76.2	-	68.6	-
23	BHT/TB	774	-	-	460	23.7	75.7	-	-	45.0
24	CC/PG	-	1052	767	-	23.3	-	101	73.5	-
25	CC/TB	-	1050	-	457	23.9	-	104	-	45.5
26	PG/TB	-	-	821	458	22.7	-	-	78.6	43.8
27	BHT/CC/PG/TB	377	519	383	230	22.6	36.3	50.0	36.9	22.1
28	BHT/CC/PG/TB	218	243	184	112	22.0	20.2	22.5	17.1	10.3

[a] Food consumption was determined over study days 0-3, 3-7, 7-10, 10-14, 14-18, 18-21, 21-24 and 24-28. Values are means of four cages for the controls (group 1) and two cages for the treated groups (groups 2 to 28). Each cage contained three rats.

[b] Calculated from body weight and food consumption data over study days 0-3, 3-7, 7-10, 10−14, 14-18, 18-21, 21-24 and 24-28.

Table 18. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on body weight

Group	Treatment	Target compound concentration (ppm in diet)				Body weight (g)[a]	
		BHT	CC	PG	TB		
1	Control	-	-	-	-	299 ± 4.8	(100%)
2	BHT	762	-	-	-	290 ± 7.1	(97%)
3	BHT	1523	-	-	-	283 ± 6.4	(95%)
4	BHT	3046	-	-	-	293 ± 6.3	(98%)
5	CC	-	1016	-	-	304 ± 5.1	(102%)
6	CC	-	2031	-	-	294 ± 6.6	(98%)
7	CC	-	4062	-	-	295 ± 11.0	(99%)
8	PG	-	-	762	-	292 ± 2.8	(97%)
9	PG	-	-	1523	-	294 ± 5.8	(98%)
10	PG	-	-	3046	-	286 ± 7.7	(96%)
11	TB	-	-	-	438	291 ± 4.5	(97%)
12	TB	-	-	-	875	294 ± 8.5	(98%)
13	TB	-	-	-	1750	256 ± 8.9	(86%)***
14	BHT/CC	1523	2031	-	-	282 ± 9.5	(94%)
15	BHT/PG	1523	-	1523	-	288 ± 4.8	(96%)
16	BHT/TB	1523	-	-	875	271 ± 6.0	(91%)**
17	CC/PG	-	2031	1523	-	295 ± 5.4	(99%)
18	CC/TB	-	2031	-	875	291 ± 6.2	(97%)
19	PG/TB	-	-	1523	875	262 ± 7.8	(87%)***
20	BHT/CC/PG/TB	762	1016	762	438	303 ± 11.9	(101%)
21	BHT/CC	762	1016	-	-	280 ± 5.9	(94%)*
22	BHT/PG	762	-	762	-	290 ± 6.9	(97%)
23	BHT/TB	762	-	-	438	300 ± 11.1	(100%)
24	CC/PG	-	1016	762	-	295 ± 5.9	(98%)
25	CC/TB	-	1016	-	438	296 ± 4.2	(99%)
26	PG/TB	-	-	762	438	290 ± 7.2	(97%)
27	BHT/CC/PG/TB	381	508	381	219	283 ± 8.5	(95%)
28	BHT/CC/PG/TB	190	254	190	109	285 ± 9.7	(95%)

[a]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats. Percentage of control values are shown in parentheses.

Values significantly different from control are: *p<0.05;**p<0.01;***p<0.001.

Table 19. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on liver weight

Group	Treatment	Target compound concentration (ppm in diet)				Liver weight (g)[a]	
		BHT	CC	PG	TB		
1	Control	-	-	-	-	11.13 ± 0.34	(100%)
2	BHT	762	-	-	-	11.94 ± 0.42	(107%)
3	BHT	1523	-	-	-	12.16 ± 0.41	(109%)
4	BHT	3046	-	-	-	14.43 ± 0.56	(130%)***
5	CC	-	1016	-	-	11.42 ± 0.59	(103%)
6	CC	-	2031	-	-	11.34 ± 0.49	(102%)
7	CC	-	4062	-	-	11.25 ± 0.64	(101%)
8	PG	-	-	762	-	10.81 ± 0.27	(97%)
9	PG	-	-	1523	-	10.96 ± 0.45	(98%)
10	PG	-	-	3046	-	10.08 ± 0.66	(91%)
11	TB	-	-	-	438	11.77 ± 0.41	(106%)
12	TB	-	-	-	875	11.27 ± 0.60	(101%)
13	TB	-	-	-	1750	9.82 ± 0.40	(88%)*
14	BHT/CC	1523	2031	-	-	12.61 ± 0.77	(113%)*
15	BHT/PG	1523	-	1523	-	12.54 ± 0.41	(113%)*
16	BHT/TB	1523	-	-	875	12.13 ± 0.59	(109%)
17	CC/PG	-	2031	1523	-	11.32 ± 0.34	(102%)
18	CC/TB	-	2031	-	875	11.47 ± 0.17	(103%)
19	PG/TB	-	-	1523	875	10.46 ± 0.50	(94%)
20	BHT/CC/PG/TB	762	1016	732	444	12.79 ± 0.75	(115%)**
21	BHT/CC	762	1016	-	-	10.97 ± 0.35	(99%)
22	BHT/PG	762	-	762	-	11.62 ± 0.51	(104%)
23	BHT/TB	762	-	-	438	12.63 ± 0.68	(113%)*
24	CC/PG	-	1016	762	-	11.33 ± 0.48	(102%)
25	CC/TB	-	1016	-	438	11.93 ± 0.36	(107%)
26	PG/TB	-	-	762	438	11.38 ± 0.59	(102%)
27	BHT/CC/PG/TB	381	508	381	219	11.48 ± 0.48	(103%)
28	BHT/CC/PG/TB	190	2545	190	109	11.42 ± 0.47	(103%)

[a]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats. Percentage of control values are shown in parentheses.

Values significantly different from control are: *p<0.05;**p<0.01;***p<0.001.

Table 21. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on hepatic microsomal protein content

Group	Treatment	Target compound concentration (ppm in diet)				Microsomal protein (mg/g liver)[a]	
		BHT	CC	PG	TB		
1	Control	-	-	-	-	36.5	± 0.7
2	BHT	762	-	-	-	38.9	± 1.4
3	BHT	1523	-	-	-	44.5	± 1.5***
4	BHT	3046	-	-	-	49.6	± 1.4***
5	CC	-	1016	-	-	37.2	± 2.1
6	CC	-	2031	-	-	37.3	± 0.8
7	CC	-	4062	-	-	36.7	± 0.8
8	PG	-	-	762	-	36.1	± 1.6
9	PG	-	-	1523	-	39.1	± 0.9
10	PG	-	-	3046	-	37.6	± 0.9
11	TB	-	-	-	438	39.4	± 1.3
12	TB	-	-	-	875	44.8	± 1.4***
13	TB	-	-	-	1750	45.2	± 1.0***
14	BHT/CC	1523	2031	-	-	46.3	± 1.6***
15	BHT/PG	1523	-	1523	-	45.3	± 1.3***
16	BHT/TB	1523	-	-	875	47.9	± 1.1***
17	CC/PG	-	2031	1523	-	37.4	± 1.5
18	CC/TB	-	2031	-	875	45.1	± 1.0***
19	PG/TB	-	-	1523	875	39.5	± 1.4
20	BHT/CC/PG/TB	762	1016	762	438	42.8	± 1.4***
21	BHT/CC	762	1016	-	-	40.2	± 2.0*
22	BHT/PG	762	-	762	-	41.4	± 1.8**
23	BHT/TB	762	-	-	438	42.0	± 1.0***
24	CC/PG	-	1016	762	-	40.0	± 1.2*
25	CC/TB	-	1016	-	438	42.2	± 0.8***
26	PG/TB	-	-	762	438	40.1	± 1.0*
27	BHT/CC/PG/TB	381	508	381	219	40.0	± 0.6*
28	BHT/CC/PG/TB	190	254	190	109	36.3	± 0.8

[a]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats.

Values significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

Table 22. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on hepatic microsomal CYP content

Group	Treatment[a]	CYP content[b]		
		(nmol/mg protein)	(nmol/g liver)	(nmol/liver weight/kg body weight)
1	Control	0.73 ± 0.02	26.7 ± 0.9	989 ± 36
2	BHT	0.76 ± 0.02	29.3 ± 1.1	1204 ± 23*
3	BHT	0.85 ± 0.03**	38.2 ± 2.5***	1646 ± 115***
4	BHT	0.99 ± 0.05***	48.9 ± 2.1***	2402 ± 109***
5	CC	0.75 ± 0.02	28.0 ± 1.9	1049 ± 76
6	CC	0.80 ± 0.03	29.9 ± 1.4	1151 ± 60
7	CC	0.77 ± 0.03	28.4 ± 1.6	1083 ± 66
8	PG	0.75 ± 0.05	27.1 ± 1.9	1007 ± 76
9	PG	0.78 ± 0.04	30.5 ± 2.1	1133 ± 80
10	PG	0.77 ± 0.03	28.7 ± 0.7	1013 ± 62
11	TB	0.77 ± 0.02	30.3 ± 1.3	1223 ± 39**
12	TB	0.81 ± 0.02*	36.3 ± 1.1***	1390 ± 66***
13	TB	0.75 ± 0.03	34.2 ± 1.8***	1307 ± 58***
14	BHT/CC	0.82 ± 0.02*	38.2 ± 1.7***	1698 ± 93***
15	BHT/PG	0.84 ± 0.02**	37.9 ± 0.4***	1650 ± 40***
16	BHT/TB	0.79 ± 0.07	37.9 ± 3.5***	1663 ± 98***
17	CC/PG	0.71 ± 0.03	26.6 ± 1.0	1019 ± 32
18	CC/TB	0.76 ± 0.03	34.4 ± 1.3***	1352 ± 36***
19	PG/TB	0.78 ± 0.03	30.8 ± 0.8*	1231 ± 47**
20	BHT/CC/PG/TB	0.79 ± 0.02	34.0 ± 1.9***	1437 ± 107***
21	BHT/CC	0.72 ± 0.02	29.0 ± 2.1	1136 ± 87
22	BHT/PG	0.78 ± 0.03	32.4 ± 2.6**	1290 ± 89***
23	BHT/TB	0.79 ± 0.03	33.2 ± 2.1**	1384 ± 68***
24	CC/PG	0.76 ± 0.01	30.3 ± 1.0	1167 ± 70*
25	CC/TB	0.78 ± 0.02	32.8 ± 0.9**	1318 ± 23***
26	PG/TB	0.78 ± 0.02	31.1 ± 1.4*	1212 ± 43**
27	BHT/CC/PG/TB	0.76 ± 0.03	30.4 ± 1.4	1237 ± 79**
28	BHT/CC/PG/TB	0.73 ± 0.03	26.6 ± 1.2	1061 ± 47

[a]For details of the dietary levels of BHT, CC, PG and TB see Table 18.
[b]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats.

Values significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

Table 25. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on hepatic CYP1A2 mRNA

Group	Treatment	Target compound concentration (ppm in diet)				CYP1A2 mRNA level (fold induction)[a,b]	
		BHT	CC	PG	TB		
1	Control	-	-	-	-	1.00	± 0.047
2	BHT	762	-	-	-	1.52	± 0.261
3	BHT	1523	-	-	-	1.31	± 0.407
4	BHT	3046	-	-	-	1.68	± 0.389
5	CC	-	1016	-	-	1.46	± 0.587
6	CC	-	2031	-	-	2.03	± 0.632
7	CC	-	4062	-	-	2.36	± 0.575*
8	PG	-	-	762	-	3.54	± 0.292***
9	PG	-	-	1523	-	3.52	± 0.368***
10	PG	-	-	3046	-	3.91	± 0.484***
11	TB	-	-	-	438	8.76	± 1.083***
12	TB	-	-	-	875	14.44	± 2.711***
13	TB	-	-	-	1750	15.13	± 2.353***
14	BHT/CC	1523	2031	-	-	3.39	± 0.630***
15	BHT/PG	1523	-	1523	-	1.19	± 0.104
16	BHT/TB	1523	-	-	875	3.86	± 0.339***
17	CC/PG	-	2031	1523	-	1.67	± 0.383
18	CC/TB	-	2031	-	875	5.27	± 0.551***
19	PG/TB	-	-	1523	875	8.14	± 1.772***
20	BHT/CC/PG/TB	762	1016	762	438	3.53	± 0.340***
21	BHT/CC	762	1016	-	-	2.07	± 0.553*
22	BHT/PG	762	-	762	-	0.74	± 0.247
23	BHT/TB	762	-	-	438	0.99	± 0.123
24	CC/PG	-	1016	762	-	0.45	± 0.118***
25	CC/TB	-	1016	-	438	0.70	± 0.169
26	PG/TB	-	-	762	438	1.15	± 0.221
27	BHT/CC/PG/TB	381	508	381	219	0.68	± 0.069
28	BHT/CC/PG/TB	190	254	190	109	0.62	± 0.198*

[a]To normalise for RNA loading, levels of CYP1A2 mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction.

[b]Results are presented as mean ± SEM for groups of 9 control (group 1) and either 5 (group 8 only) or 6 (groups 2-7 and 9-28) treated rats.

Values significantly different from control are: *p<0.05; ***p<0.001.

Table 26. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on hepatic CYP2B1 mRNA

Group	Treatment	Target compound concentration (ppm in diet)				CYP2B1 mRNA level (fold induction)[a,b]	
		BHT	CC	PG	TB		
1	Control	-	-	-	-	1.00	± 0.043
2	BHT	762	-	-	-	57.88	± 13.295***
3	BHT	1523	-	-	-	555.47	± 154.236***
4	BHT	3046	-	-	-	1827.03	± 750.977***
5	CC	-	1016	-	-	10.67	± 4.406***
6	CC	-	2031	-	-	3.82	± 1.619
7	CC	-	4062	-	-	1.50	± 0.517
8	PG	-	-	762	-	0.66	± 0.100
9	PG	-	-	1523	-	3.06	± 1.057
10	PG	-	-	3046	-	0.71	± 0.102
11	TB	-	-	-	438	1.08	± 0.412
12	TB	-	-	-	875	1.82	± 0.462
13	TB	-	-	-	1750	9.79	± 2.251***
14	BHT/CC	1523	2031	-	-	396.86	± 47.150***
15	BHT/PG	1523	-	1523	-	323.76	± 54.818***
16	BHT/TB	1523	-	-	875	934.18	± 344.114***
17	CC/PG	-	2031	1523	-	46.74	± 26.243***
18	CC/TB	-	2031	-	875	11.89	± 6.194***
19	PG/TB	-	-	1523	875	9.37	± 3.778***
20	BHT/CC/PG/TB	762	1016	762	438	109.63	± 25.240***
21	BHT/CC	762	1016	-	-	194.54	± 40.423***
22	BHT/PG	762	-	762	-	84.93	± 31.419***
23	BHT/TB	762	-	-	438	56.03	± 11.991***
24	CC/PG	-	1016	762	-	6.11	± 1.714**
25	CC/TB	-	1016	-	438	2.32	± 0.894
26	PG/TB	-	-	762	438	0.96	± 0.396
27	BHT/CC/PG/TB	381	508	381	219	32.33	± 20.138***
28	BHT/CC/PG/TB	190	254	190	109	14.07	± 6.374***

[a]To normalise for RNA loading, levels of CYP2B1 mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction.

[b]Results are presented as mean ± SEM for groups of 9 control (group 1) and either 5 (group 8 only) or 6 (groups 2-7 and 9-28) treated rats.

Values significantly different from control are: **p<0.01; ***p<0.001.

Table 27. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on hepatic cytosolic protein content

Group	Treatment	Target compound concentration (ppm in diet)				Cytosolic protein (mg/g liver)[a]
		BHT	CC	PG	TB	
1	Control	-	-	-	-	106.9 ± 2.5
2	BHT	762	-	-	-	101.3 ± 2.9
3	BHT	1523	-	-	-	103.5 ± 4.9
4	BHT	3046	-	-	-	98.5 ± 2.4
5	CC	-	1016	-	-	104.0 ± 3.9
6	CC	-	2031	-	-	114.5 ± 10.0
7	CC	-	4062	-	-	111.1 ± 4.5
8	PG	-	-	762	-	108.5 ± 1.6
9	PG	-	-	1523	-	106.6 ± 4.2
10	PG	-	-	3046	-	103.3 ± 3.7
11	TB	-	-	-	438	100.9 ± 2.8
12	TB	-	-	-	875	108.7 ± 3.1
13	TB	-	-	-	1750	109.4 ± 3.5
14	BHT/CC	1523	2031	-	-	99.8 ± 4.2
15	BHT/PG	1523	-	1523	-	99.1 ± 3.0
16	BHT/TB	1523	-	-	875	101.9 ± 2.9
17	CC/PG	-	2031	1523	-	102.9 ± 3.9
18	CC/TB	-	2031	-	875	106.2 ± 3.1
19	PG/TB	-	-	1523	875	103.6 ± 3.3
20	BHT/CC/PG/TB	762	1016	762	438	104.0 ± 4.9
21	BHT/CC	762	1016	-	-	99.7 ± 1.6
22	BHT/PG	762	-	762	-	99.6 ± 2.5
23	BHT/TB	762	-	-	438	100.2 ± 2.0
24	CC/PG	-	1016	762	-	102.1 ± 2.6
25	CC/TB	-	1016	-	438	104.0 ± 3.5
26	PG/TB	-	-	762	438	98.2 ± 3.1
27	BHT/CC/PG/TB	381	508	381	219	100.4 ± 4.1
28	BHT/CC/PG/TB	190	254	190	109	105.1 ± 2.9

[a]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats.

All values not significantly different from control (all $p>0.05$).

Table 28. Effect of treatment of rats for 28 days with diets containing various combinations of BHT, CC, PB and TB on hepatic GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) as substrate

Group	Treatment[a]	GST activity[b]								
		(μmol/min/ mg protein)			(μmol/min/g liver)			(μmol/min/liver kg body weight)		
1	Control	2.10	± 0.11		225	± 13		8401	± 406	
2	BHT	2.47	± 0.18		270	± 13		11148	± 651	
3	BHT	4.23	± 0.25	***	438	± 33	***	18742	± 1266	***
4	BHT	5.51	± 0.44	***	550	± 33	***	27069	± 1942	***
5	CC	2.19	± 0.19		224	± 21		8295	± 661	
6	CC	2.13	± 0.18		236	± 14		9019	± 433	
7	CC	1.96	± 0.12		220	± 11		8349	± 374	
8	PG	2.30	± 0.27		254	± 23		9337	± 661	
9	PG	2.26	± 0.33		241	± 25		8932	± 922	
10	PG	2.37	± 0.13		240	± 19		8378	± 691	
11	TB	2.75	± 0.20		279	± 16		11231	± 526	
12	TB	2.92	± 0.19	*	312	± 27	*	11786	± 662	*
13	TB	4.32	± 0.41	***	437	± 48	***	16766	± 1935	***
14	BHT/CC	4.51	± 0.69	***	444	± 52	***	19747	± 2422	***
15	BHT/PG	4.58	± 0.53	***	468	± 34	***	20508	± 1889	***
16	BHT/TB	4.61	± 0.11	***	425	± 39	***	18873	± 1654	***
17	CC/PG	2.66	± 0.10		270	± 4		10384	± 444	
18	CC/TB	3.62	± 0.32	***	383	± 40	***	14979	± 1411	***
19	PG/TB	3.29	± 0.28	**	334	± 23	**	13316	± 865	**
20	BHT/CC/PG/TB	4.55	± 0.56	***	446	± 49	***	18904	± 2387	***
21	BHT/CC	3.18	± 0.34	**	314	± 34	*	12240	± 1314	*
22	BHT/PG	2.86	± 0.26		285	± 27		11346	± 942	
23	BHT/TB	4.78	± 0.53	***	479	± 53	***	20154	± 2392	***
24	CC/PG	2.29	± 0.12		232	± 12		8853	± 376	
25	CC/TB	3.23	± 0.43	**	350	± 28	***	14127	± 1282	***
26	PG/TB	2.89	± 0.34	*	285	± 29		11096	± 1074	
27	BHT/CC/PG/TB	3.00	± 0.26	*	308	± 19	*	12491	± 928	**
28	BHT/CC/PG/TB	2.94	± 0.15	*	302	± 22	*	12052	± 790	*

[a]For details of the dietary levels of BHT, CC, PG and TB see Table 27.
[b]Results are presented as mean ± SEM for groups of 12 control (group 1) and 6 treated (groups 2 to 28) rats.

Values significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

Table 30a. Effect of treatment of rats for 28 days with various dietary levels of BHT, CC, PG and TB and hepatic GSTM2 and GSTP1 mRNA levels

Group	Treatment[a]	GST mRNA level (fold induction)[b,c]			
		GSTM2		GSTP1	
1	Control	1.00	± 0.034	1.00	± 0.040
2	BHT	1.26	± 0.238	0.98	± 0.239
3	BHT	1.89	± 0.474	2.54	± 1.026
4	BHT	2.00	± 0.330	1.22	± 0.377
5	CC	1.18	± 0.287	3.52	± 1.974**
6	CC	0.91	± 0.064	2.37	± 0.571
7	CC	0.97	± 0.147	0.98	± 0.467
8	PG	0.48	± 0.064	0.66	± 0.095
9	PG	0.69	± 0.071	1.48	± 0.468
10	PG	0.54	± 0.068	0.68	± 0.085
11	TB	1.08	± 0.412	0.92	± 0.064
12	TB	1.82	± 0.462	0.99	± 0.121
13	TB	9.79	± 2.251***	2.42	± 0.740

[a]For details of the dietary levels of BHT, CC, PG and TB see Table 27.
[b]To normalise for RNA loading, levels of GST mRNAs were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction.
[c]Results are presented as mean ± SEM for groups of 9 control (group 1) and either 5 (group 8 only) or 6 (groups 2-7 and 10-13) treated rats.

Values significantly different from control are: **p<0.01; ***p<0.001.

Table 31. Effect of treatment of rat hepatocytes for 72 hours with TB, PG and cadmium chloride on cytotoxicity employing the MTT assay

Hepatocyte treatment	MTT cytotoxicity[a]	Percentage of control
Control (DMSO only)	0.607 ± 0.021	(100)
TB 0.5 μM	0.636 ± 0.049	105
TB 2 μM	0.664 ± 0.050	109
TB 5 μM	0.594 ± 0.046	98
TB 20 μM	0.584 ± 0.041	96
TB 50 μM	0.607 ± 0.062	100
TB 100 μM	0.563 ± 0.032	93
TB 200 μM	0.468 ± 0.043	77
PG 0.5 μM	0.664 ± 0.097	109
PG 2 μM	0.627 ± 0.082	103
PG 5 μM	0.629 ± 0.072	104
PG 20 μM	0.716 ± 0.081	118
PG 50 μM	0.601 ± 0.101	99
Cadmium chloride 20 μM	0.005 ± 0.001	1***

[a]Results are presented as mean ± SEM of 4 experiments in units of absorbance at 570nm/30 minute incubation.

Values significantly different from control are: ***p<0.001.

Table 32. Effect of treatment of rat hepatocytes for 72 hours with BHT, CC and menadione on cytotoxicity employing the MTT assay

Hepatocyte treatment	MTT cytotoxicity[a]	Percentage of control
Control (DMSO only)	0.621 ± 0.032	(100)
BHT 0.5 μM	0.653 ±0.024	105
BHT 2 μM	0.630 ± 0.036	101
BHT 5 μM	0.706 ± 0.028	114
BHT 20 μM	0.675 ± 0.026	109
BHT 50 μM	0.570 ± 0.010	92
BHT 100 μM	0.620 ± 0.030	100
BHT 200 μM	0.575 ± 0.099	93
CC 0.5 μM	0.638 ± 0.044	103
CC 2 μM	0.632 ± 0.043	102
CC 5 μM	0.627 ± 0.053	101
CC 20 μM	0.553 ± 0.049	89
CC 50 μM	0.188 ± 0.110	30***
Menadione 50 μM	0.011 ± 0.003	2***

[a]Results are presented as mean ± SEM of 4 experiments in units of absorbance at 570nm/30 minute incubation.

Values significantly different from control are: ***p<0.001.

Table 33. Effect of treatment of rat hepatocytes for 72 hours with TB, BHT, PG and CC on CYP1A2, CYP2B1 and CYP3A1 mRNA levels

Hepatocyte treatment	CYP mRNA (fold induction)[a]		
	CYP1A2	CYP2B1	CYP3A1
Control (DMSO only)	1.00	1.00	1.00
TB 2 μM	1.52 ± 0.30	0.59 ± 0.10	0.65 ± 0.07
TB 5 μM	1.61 ± 0.31	0.79 ± 0.18	0.70 ± 0.08
TB 20 μM	2.25 ± 0.48	1.50 ± 0.26	0.74 ± 0.05
TB 50 μM	8.92 ± 2.10	3.11 ± 0.44	1.31 ± 0.27
TB 100 μM	34.34 ± 11.68	3.59 ± 0.26	1.75 ± 0.23
TB 200 μM	50.29 ± 6.47	2.49 ± 0.58	2.28 ± 0.22
BHT 2 μM	0.89 ± 0.25	0.75 ± 0.14	0.84 ± 0.08
BHT 5 μM	0.99 ± 0.20	0.91 ± 0.18	0.83 ± 0.12
BHT 20 μM	0.74 ± 0.15	1.65 ± 0.35	0.84 ± 0.06
BHT 50 μM	0.56 ± 0.13	3.00 ± 0.44	1.19 ± 0.15
BHT 100 μM	0.35 ± 0.04	5.70 ± 0.58	1.89 ± 0.50
BHT 200 μM	0.42 ± 0.10	8.00 ± 2.91	2.21 ± 0.32
PG 0.5 μM	0.96 ± 0.20	1.71 ± 0.25	1.44 ± 0.27
PG 2 μM	1.26 ± 0.23	1.33 ± 0.28	1.37 ± 0.19
PG 5 μM	1.32 ± 0.10	1.36 ± 0.29	1.28 ± 0.24
PG 20 μM	1.02 ± 0.17	1.88 ± 0.83	1.46 ± 0.43
CC 0.5 μM	0.81 ± 0.18	0.70 ± 0.15	0.72 ± 0.17
CC 2 μM	0.83 ± 0.22	0.95 ± 0.16	0.84 ± 0.15
CC 5 μM	0.81 ± 0.15	1.33 ± 0.23	0.86 ± 0.16
CC 20 μM	0.92 ± 0.23	2.56 ± 0.74	0.92 ± 0.27

[a]Results are presented as mean ± SEM of either 4 (CYP2B1) or 5 (CYP1A2 and CYP3A1) experiments. For other details see Table 34.

Table 34. Effect of treatment of rat hepatocytes for 72 hours with BNF, NaPB and PCN on CYP1A2, CYP2B1 and CYP3A1 mRNA levels

Hepatocyte treatment	CYP mRNA (fold induction)[a]		
	CYP1A2	CYP2B1	CYP3A1
Control (DMSO only)	1.00	1.00	1.00
BNF 0.05 μM	12.58 ± 0.93	0.73 ± 0.18	0.72 ± 0.06
BNF 0.2 μM	55.47 ± 9.83	0.45 ± 0.13	0.67 ± 0.17
BNF 2 μM	172.16 ± 32.47	0.76 ± 0.52	0.89 ± 0.41
NaPB 50 μM	1.04 ± 0.26	25.70 ± 9.25	1.78 ± 0.73
NaPB 200 μM	1.26 ± .040	39.04 ± 10.70	3.96 ± 2.44
NaPB 500 μM	1.27 ± 0.22	39.40 ± 6.84	6.98 ± 4.10
PCN 0.5 μM	1.05 ± 0.30	0.57 ± 0.08	20.96 ± 17.79
PCN 2 μM	0.78 ± 0.20	0.54 ± 0.10	26.44 ± 21.16
PCN 5 μM	1.00 ± 0.49	0.63 ± 0.24	31.53 ± 26.43
PCN 20 μM	1.83 ± 1.20	1.29 ± 0.77	44.35 ± 38.11

[a]Results are presented as mean ± SEM of either 4 (CYP2B1) or 5 (CYP1A2 and CYP3A1) experiments. For the data shown in Tables 33 and 34, in each experiment up to 4 control pools (each of 4 wells) and up to 2 pools (each of 4 wells) for all concentrations of TB, BHT, PG, CC, BNF, NaPB and PCN were assayed.

Table 35. Effect of treatment of rat hepatocytes for 72 hours with TB, PG and NaPB on 7-ethoxyresorufin O-deethylase and BFC O-debenzylase activities

Hepatocyte treatment	7-Ethoxyresorufin O-deethylase[a]		BFC O-debenzylase[a]	
	(pmol/min/mg protein)	Percentage of control	(pmol/min/mg protein)	Percentage of control
Control (DMSO only)	37	(100)	56 ± 11	(100)
TB 0.5 μM	24	65	35	63
TB 2 μM	19	52	49 ± 19	87
TB 5 μM	14	38	43 ± 17	77
TB 20 μM	17	47	66 ± 23	119
TB 50 μM	28	77	124 ± 56	223
TB 100 μM	51	140	173 ± 75	310
TB 200 μM	85	231	222 ± 60	399
PG 0.5 μM	30	81	35	62
PG 2 μM	22	60	30	54
PG 5 μM	23	62	33	59
PG 20 μM	29	80	21	37
PG 50 μM	21	57	23	41
NaPB 200 μM	86	236	399	716
NaPB 500 μM	103	280	378 ± 55	678

[a]Results are presented as either the mean of 2 experiments or the mean ± SEM of 3 experiments. In each experiment up to 12 wells for controls (DMSO only treated) and up to 6 wells for all concentrations of TB, PG and NaPB were assayed.

Table 36. Effect of treatment of rat hepatocytes for 72 hours with BHT, CC and BNF on 7-ethoxyresorufin O-deethylase and BFC O-debenzylase activities

Hepatocyte treatment	7-Ethoxyresorufin O-deethylase[a]		BFC O-debenzylase[a]	
	(pmol/min/mg protein)	Percentage of control	(pmol/min/mg protein)	Percentage of control
Control (DMSO only)	36	(100)	55 ± 9	(100)
BHT 0.5 μM	33	92	44	79
BHT 2 μM	40	111	60 ± 14	109
BHT 5 μM	41	113	68 ± 17	123
BHT 20 μM	51	141	96 ± 31	173
BHT 50 μM	56	156	123 ± 34	223
BHT 100 μM	39	108	135 ± 27	244
BHT 200 μM	34	94	105 ± 17	189
CC 0.5 μM	43	119	54	97
CC 2 μM	31	87	45	82
CC 5 μM	26	71	34	62
CC 20 μM	13	37	15	26
CC 50 μM	9	25	12	22
BNF 0.2 μM	312	864	124	223
BNF 2 μM	363	1004	150 ± 16	270

[a]Results are presented as either the mean of 2 experiments or the mean ± SEM of 3 experiments. In each experiment up to 12 wells for controls (DMSO only treated) and up to 6 wells for all concentrations of BHT, CC and BNF were assayed.

Table 37. Initial design of rat *in vitro* mixtures study with BHT, CC, PG and TB

Group[a]	Food additive concentration in culture medium (µM)			
	BHT	TB	PG	CC
Control	0	0	0	0
1	0	0	0	2
2	0	0	0	15
3	0	0	2	0
4	0	0	20	0
5	5	0	0	0
6	100	0	0	0
7	0	20	0	0
8	0	100	0	0
9	100	100	0	0
10	100	0	20	0
11	100	0	0	15
12	0	100	20	0
13	0	100	0	15
14	0	0	20	15
15	5	20	2	2
16	5	100	2	2
17	100	20	2	2
18	100	100	2	2
19	5	20	20	2
20	5	100	20	2
21	100	20	20	2
22	100	100	20	2
23	5	20	2	15
24	5	100	2	15
25	100	20	2	15
26	100	100	2	15
27	5	20	20	15
28	5	100	20	15
29	100	20	20	15
30	100	100	20	15

[a]Groups 1 to 8 are individual compound concentrations.
Groups 9 to 14 are binary mixtures (high concentrations only).
Groups 15 to 30 are quaternary mixtures (low and high concentrations).

Table 39. Effect of treatment of rat hepatocytes for 72 hours with some food additive binary mixtures on cytotoxicity employing the MTT assay

Hepatocyte treatment[a]	MTT cytotoxicity[b]	Percentage of control
Control (DMSO only)	0.564 ± 0.040	(100)
23	0.393 ± 0.093	70
24	0.292 ± 0.093*	52
25	0.296 ± 0.130*	53
26	0.142 ± 0.070**	25
27	0.234 ± 0.076**	42
28	0.202 ± 0.077**	36
29	0.150 ± 0.080**	27
30	0.126 ± 0.073**	22

[a]Treatments refer to food additive binary mixtures (Groups 23 to 30) shown in Table 37.
[b]Results are presented as mean ± SEM of 4 experiments in units of absorbance at 570 nm/30 minute incubation.

Values significantly different from control are: *$p<0.05$; **$p<0.01$.

Table 40. Effect of treatment of rat hepatocytes for 72 hours with individual food additives and food additive mixtures on cytotoxicity employing the MTT assay

Hepatocyte treatment[a]	MTT cytotoxicity[b]	Percentage of control
Control (DMSO only)	0.549 ± 0.084	(100)
1	0.613 ± 0.072	112
31	0.689 ± 0.036	126
2	0.643 ± 0.052	117
3	0.579 ± 0.070	106
4	0.619 ± 0.065	113
5	0.628 ± 0.035	114
6	0.620 ± 0.035	113
7	0.487 ± 0.038	89
8	0.525 ± 0.032	96
9	0.506 ± 0.025	92
10	0.738 ± 0.038*	134
11	0.562 ± 0.078	102
12	0.469 ± 0.064	85
13	0.440 ± 0.053	80
14	0.274 ± 0.088**	50
15	0.531 ± 0.061	97
16	0.502 ± 0.033	91
17	0.580 ± 0.069	106
18	0.507 ± 0.074	92
19	0.571 ± 0.076	104
20	0.546 ± 0.048	100
21	0.593 ± 0.084	108
22	0.503 ± 0.075	92
33	0.611 ± 0.044	111
34	0.462 ± 0.060	84
35	0.512 ± 0.076	93
36	0.463 ± 0.065	84
37	0.509 ± 0.094	93
38	0.497 ± 0.087	91
39	0.444 ± 0.104	81
40	0.358 ± 0.097*	65
$CdCl_2$ 20 µM	0.009 ± 0.001***	2
Menadione 50 µM	0.010 ± 0.002***	2

[a]Treatments refer to individual food additive and food additive mixtures (Groups 1, 31, 2 to 22 and 33 to 40) shown in Table 38.

[b]Results are presented as mean ± SEM of 4 experiments in units of absorbance at 570nm/30 minute incubation.

Values significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

Table 41. Effect of treatment of rat hepatocytes with individual food additives and food additive mixtures on CYP1A2 mRNA levels

Group[a]	Hepatocyte experiment				CYP1A2 mRNA[b]	
	A	B	C	D	Mean	± SEM
Control	1.00	1.00	1.00	1.00	1.00	-
1	1.21	1.01	0.59	1.25	1.02	0.15
31	1.60	2.26	0.61	0.72	1.30	0.39
2	0.54	1.81	0.52	0.74	0.90	0.31
3	1.63	1.08	1.09	1.47	1.32	0.14
4	0.91	0.74	0.67	0.80	0.78	0.05
5	1.00	1.77	0.48	1.32	1.14	0.27
6	0.62	1.13	3.35	0.80	1.47	0.63
7	2.28	2.76	6.64	2.72	3.60	1.02
8	24.52	19.08	28.68	25.15	24.36	1.98
9	17.75	16.77	19.86	17.44	17.95	0.67
10	1.15	1.75	2.46	1.70	1.76	0.27
11	0.36	1.58	0.68	0.18	0.70	0.31
12	15.84	28.36	31.12	26.37	25.42	3.34
13	13.23	24.99	26.43	25.17	22.45	3.09
14	2.81	ND	2.75	2.75	2.77	0.02
15	1.71	1.79	1.54	1.66	1.67	0.05
16	16.57	19.46	29.88	27.10	23.25	3.13
17	0.77	1.27	1.42	1.10	1.14	0.14
18	9.87	9.64	11.21	11.81	10.63	0.52
19	7.19	3.66	2.47	2.41	3.93	1.12
20	14.50	24.83	50.77	28.35	29.61	7.64
21	0.83	4.24	1.69	1.78	2.13	0.73
22	3.93	15.66	23.74	9.56	13.22	4.25
33	1.82	15.01	2.90	2.96	5.67	3.12
34	23.00	67.99	64.12	21.65	44.19	12.65
35	1.25	21.75	2.01	1.29	6.58	5.06
36	6.09	35.97	12.58	15.90	17.64	6.44
37	0.92	5.83	3.16	5.42	3.83	1.13
38	8.92	58.77	43.01	33.97	36.17	10.43
39	1.14	6.21	1.92	2.78	3.01	1.12
40	4.43	20.46	9.56	5.71	10.04	3.64

[a]See Table 38 for food additive culture medium concentrations.
[b]Results are presented as mean ± SEM of four replicate experiments (experiments A, B, C and D) in units of fold induction.

Table 42. Effect of treatment of rat hepatocytes with individual food additives and food additive mixtures on CYP2B1 mRNA levels

Group[a]	Hepatocyte experiment				CYP2B1 mRNA[b]	
	A	B	C	D	Mean	± SEM
Control	1.00	1.00	1.00	1.00	1.00	-
1	0.77	0.42	0.56	0.68	0.61	0.08
31	0.54	0.34	0.88	0.74	0.62	0.12
2	0.53	0.71	1.09	0.55	0.72	0.13
3	1.07	0.86	1.08	0.72	0.93	0.09
4	2.02	1.37	1.49	0.86	1.43	0.24
5	1.17	1.00	1.08	1.53	1.19	0.12
6	3.35	2.88	2.62	7.42	4.07	1.13
7	0.93	0.80	1.41	1.57	1.18	0.19
8	0.69	1.15	1.33	1.97	1.29	0.27
9	0.75	1.35	3.56	5.28	2.74	1.04
10	5.02	3.15	1.28	1.57	2.75	0.86
11	0.47	0.45	2.96	1.97	1.46	0.61
12	1.36	3.59	1.84	2.49	2.32	0.48
13	0.66	2.46	0.91	1.19	1.31	0.40
14	1.03	ND	0.62	1.19	0.95	0.15
15	1.77	1.13	0.96	1.49	1.34	0.18
16	0.70	1.30	1.05	1.71	1.19	0.21
17	0.72	0.95	2.67	5.33	2.42	1.06
18	0.35	0.69	1.83	2.59	1.36	0.52
19	3.02	1.79	0.98	1.56	1.83	0.43
20	1.50	2.10	1.86	2.26	1.93	0.16
21	2.30	2.99	2.56	7.59	3.86	1.25
22	1.95	3.76	3.29	3.68	3.17	0.42
33	0.71	1.79	1.64	0.98	1.28	0.26
34	1.00	2.62	1.36	1.35	1.58	0.36
35	0.26	1.10	0.89	2.79	1.26	0.54
36	0.57	0.70	1.07	2.10	1.11	0.35
37	2.63	3.79	1.16	0.99	2.14	0.66
38	2.99	9.68	2.90	3.72	4.82	1.63
39	4.74	3.08	1.27	0.93	2.51	0.88
40	14.20	4.64	1.89	1.26	5.50	2.99

[a]See Table 38 for food additive culture medium concentrations.
[b]Results are presented as mean ± SEM of four replicate experiments (experiments A, B, C and D) in units of fold induction.

Table 44. Effect of treatment of rat hepatocytes with BNF and NaPB on CYP1A2 and CYP2B1 mRNA levels and BFC O-debenzylase activity

Treatment	CYP mRNA (fold induction)[a]		BFC O-debenzylase activity[a,b]
	CYP1A2	CYP2B1	
Control	1.0	1.0	26 ± 6
BNF 0.05 µM	22.9 ± 15.9***	1.0 ± 0.2	87 ± 15**
BNF 0.2 µM	39.5 ± 11.9***	0.5 ± 0.1	112 ± 20***
BNF 2 µM	172.2 ± 51.6***	0.4 ± 0.1	135 ± 29***
NaPB 25 µM	1.1 ± 0.1	19.5 ± 3.7***	205 ± 81**
NaPB 100 µM	2.1 ± 0.7	33.5 ± 5.7***	216 ± 75**
NaPB 250 µM	1.6 ± 0.5	60.6 ± 11.0***	230 ± 71**

[a]Results are presented as mean ± SEM of 4 experiments.
[b]Units pmol/min/mg hepatocyte protein.

Values significantly different from control are: **p<0.01; ***p<0.001

Table 45. Clinical information on the donors of liver samples for the preparation of human hepatocytes

Hepatocyte preparation[a,b]	Ethnic origin	Age	Sex	Smoker	Cell viability (%)[c]
H1	Caucasian	60	Female	No	-[d]
H2	Caucasian	77	Male	Yes	77.0
H3	Caucasian	68	Female	Yes	76.0
H4	Unknown[d]	57	Male	Yes	80.0
H5	Unknown[d]	58	Male	Yes	78.2
H6	Caucasian	57	Male	Yes	80.5
H7	Caucasian	70	Female	Unknown[d]	84.2
H8	Caucasian	71	Female	No	77.2
H9	Caucasian	70	Male	No	80.4
H10	Caucasian	54	Male	Yes	81.9
H11	Caucasian	71	Female	No	78.8

[a]Ethical approval and donor consent was obtained for the removal of liver tissue from subjects undergoing liver resections. Hepatocytes were isolated from liver samples by UK HTB (De Montfort University, Leicester, UK) and plated out in 96-well collagen coated plates and cultured overnight in the UK HTB laboratory. The 96-well plates of human hepatocytes were then transported to either BIBRA or LFI.

[b]Hepatocyte preparations were used for MTT cytotoxicity, enzyme activity and mRNA studies as described in the text.

[c]Cell viability at isolation in UK HTB laboratories.

[d]Information not supplied by UK HTB.

Table 46. Effect of treatment of human hepatocytes for 72 hours with TB, BHT, PG and CC on cytotoxicity employing the MTT assay

Hepatocyte treatment	MTT cytotoxicity[a]	Percentage of control
Control (DMSO only)	0.489	(100)
TB 5 μM	0.543	111
TB 20 μM	0.489	100
TB 50 μM	0.534	109
TB 100 μM	0.496	101
TB 200 μM	0.417	85
BHT 5 μM	0.532	109
BHT 20 μM	0.530	108
BHT 50 μM	0.546	112
BHT 100 μM	0.545	112
BHT 200 μM	0.526	108
PG 5 μM	0.534	109
PG 20 μM	0.512	105
PG 50 μM	0.479	98
PG 100 μM	0.275	56
PG 200 μM	0.133	27
CC 2 μM	0.512	105
CC 5 μM	0.561	115
CC 20 μM	0.535	109
CC 50 μM	0.210	43
$CdCl_2$ 20 μM	0.010	2
Menadione 50 μM	0.016	3

[a]Results are presented as mean of 2 experiments in units of absorbance at 570 nm/30 minute incubation.

Table 47. Effect of treatment of human hepatocytes for 72 hours with TB, BHT, PG and CC on CYP1A2, CYP2B6 and CYP3A4 mRNA levels

Hepatocyte treatment	CYP mRNA (fold induction)[a]		
	CYP1A2	CYP2B6	CYP3A4
Control (DMSO only)	1.00	1.00	1.00
TB 0.5 μM	3.15	0.86	1.36
TB 2 μM	6.23 ± 1.97	1.09 ± 0.22	1.47 ± 0.31
TB 5 μM	8.96 ± 2.80	1.38 ± 0.27	1.67 ± 0.47
TB 20 μM	16.63 ± 4.79	3.04 ± 1.23	1.39 ± 0.35
TB 50 μM	30.78 ± 1.91	6.03 ± 2.23	2.35 ± 0.16
TB 100 μM	49.53 ± 4.06	11.90 ± 3.84	4.51 ± 0.63
TB 200 μM	52.82 ± 2.43	16.63 ± 5.62	9.96 ± 1.55
BHT 2 μM	0.55 ± 0.10	1.40 ± 0.39	2.09 ± 0.95
BHT 5 μM	0.51 ± 0.07	1.82 ± 0.76	2.58 ± 1.18
BHT 20 μM	0.76 ± 0.21	2.27 ± 0.97	3.31 ± 2.10
BHT 50 μM	1.23 ± 0.41	5.63 ± 2.71	7.18 ± 4.78
BHT 100 μM	3.29 ± 2.42	11.40 ± 5.92	7.83 ± 2.11
BHT 200 μM	3.17 ± 2.09	12.81 ± 6.96	8.19 ± 2.23
PG 5 μM	0.59 ± 0.09	1.54 ± 0.36	1.45 ± 0.25
PG 20 μM	0.60 ± 0.17	2.17 ± 0.67	1.79 ± 0.26
PG 50 μM	1.46 ± 0.36	5.09 ± 1.83	2.69 ± 0.92
PG 100 μM	2.83	6.03	1.96
PG 200 μM	2.29 ± 1.59	2.15 ± 1.04	0.98 ± 0.36
CC 2 μM	0.69	0.93	1.36
CC 5 μM	1.03 ± 0.26	1.04 ± 0.37	1.15 ± 0.19
CC 20 μM	1.18 ± 0.16	1.26 ± 0.36	1.60 ± 0.23
CC 50 μM	1.42 ± 0.22	1.49 ± 0.92	1.28 ± 0.69
CC 100 μM	1.84	0.74	1.45

[a]Results are presented as either mean of 2 experiments or mean ± SEM of 3 experiments. For other details see Table 48.

Table 48. Effect of treatment of human hepatocytes for 72 hours with BNF, NaPB and RIF on CYP1A2, CYP2B6 and CYP3A4 mRNA levels

Hepatocyte treatment	CYP mRNA (fold induction)[a]		
	CYP1A2	CYP2B6	CYP3A4
Control (DMSO only)	1.00	1.00	1.00
BNF 10 μM	88.79 ± 10.19	n.d.[b]	n.d.
BNF 50 μM	129.4 ± 9.66	n.d.	n.d
NaPB 200 μM	n.d	6.60 ± 0.66	9.85 ± 0.62
NaPB 1000 μM	n.d	20.97 ± 2.44	23.20 ± 3.33
RIF 10 μM	n.d	9.95 ± 3.30	27.41 ± 3.94
RIF 50 μM	n.d	12.55 ± 3.20	67.55 ± 17.03

[a]Results are presented as mean ± SEM of 3 experiments. For the data shown in Tables 47 and 48, in each experiment up to 4 control pools (each of 4 wells) and up to 2 pools (each of 4 wells) for all concentrations of TB, BHT, PG, CC, BNF, NaPB and RIF were assayed.

[b] n.d.; not determined.

Table 49. Effect of treatment of human hepatocytes with food additives on 7-ethoxyresorufin O-deethylase activity

Treatment	7-Ethoxyresorufin O-deethylase[a]	Percentage of control
Control	0.55 ± 0.08[b]	(100)
TB 20 µM	1.60 ± 0.35	291
TB 50 µM	2.77 ± 0.56***	504
TB 100 µM	3.71 ± 0.43***	675
TB 200 µM	2.89 ± 0.35***	526
BNF 10 µM	14.35 ± 1.29***	2609
Control	0.56 ± 0.16[c]	(100)
BHT 20 µM	0.83 ± 0.17	148
BHT 50 µM	0.87 ± 0.35	155
BHT 100 µM	0.87 ± 0.30	155
BHT 200 µM	0.90 ± 0.26	161
CC 20 µM	0.77 ± 0.28	138
PG 50 µM	0.66 ± 0.18	118

[a]7-Ethoxyresorufin O-deethylase activity in units of pmol/min/mg hepatocyte protein.
[b]Results are presented as mean ± SEM of 4 to 6 experiments.
[c]Results are presented as mean ± SEM of 3 experiments.

Values significantly different from control are: ***p<0.001.

Table 51. Effect of treatment of human hepatocytes for 72 hours with individual food additives and food additive mixtures on cytotoxicity employing the MTT assay

Hepatocyte treatment[a]	MTT Cytotoxicity[b]	Percentage of control
Control (DMSO only)	0.573 ± 0.069	(100)
2	0.633 ± 0.081	111
3	0.600 ± 0.074	105
4	0.620 ± 0.054	108
5	0.594 ± 0.055	104
6	0.597 ± 0.040	104
7	0.577 ± 0.060	101
8	0.521 ± 0.060	91
9	0.535 ± 0.055	93
10	0.560 ± 0.058	98
11	0.604 ± 0.072	105
12	0.590 ± 0.057	103
13	0.553 ± 0.056	97
14	0.592 ± 0.079	103
15	0.612 ± 0.063	107
16	0.597 ± 0.063	104
17	0.622 ± 0.066	109
18	0.575 ± 0.072	100
19	0.583 ± 0.057	102
20	0.575 ± 0.063	100
21	0.606 ± 0.057	106
22	0.602 ± 0.063	105
23	0.616 ± 0.062	108
24	0.599 ± 0.061	105
25	0.602 ± 0.066	105
26	0.600 ± 0.073	105
27	0.606 ± 0.064	106
$CdCl_2$ 20 µM	0.014 ± 0.003***	2
Menadione 50 µM	0.024 ± 0.008***	4

[a]Treatments refer to individual food additive and food additive mixtures (Groups 2 to 27) shown in Table 50. Group 1 was not studied.
[b]Results are presented as mean ± SEM of 4 experiments in units of absorbance at 570 nm/30 minute incubation.

Values significantly different from control are: ***p<0.001.

Table 52. Effect of treatment of human hepatocytes with individual food additives and food additive mixtures on CYP1A2 mRNA levels

Group[a]	Hepatocyte experiment				CYP1A2 mRNA[b]	
	A	B	C	D	Mean	± SEM
Control	1.00	1.00	1.00	1.00	1.00	-
1	0.62	0.64	0.95	1.69	0.97	0.25
2	0.66	0.81	1.17	1.44	1.02	0.18
3	0.98	0.82	1.10	1.57	1.12	0.16
4	19.38	23.55	33.94	36.70	28.39	4.13
5	23.01	65.49	37.19	57.46	45.79	9.65
6	37.67	99.03	108.53	104.44	87.42	16.70
7	1.35	1.61	1.35	1.34	1.41	0.07
8	0.74	1.31	1.63	1.00	1.17	0.19
9	0.44	0.67	1.19	0.87	0.79	0.16
10	0.73	1.00	2.28	0.96	1.24	0.35
11	1.23	1.33	1.04	1.11	1.18	0.07
12	0.87	0.91	1.55	1.26	1.15	0.16
13	26.20	32.13	19.31	35.38	28.25	3.54
14	4.11	1.86	2.21	3.86	3.01	0.57
15	1.68	0.81	1.39	1.63	1.38	0.20
16	7.73	22.50	14.57	17.36	15.54	3.08
17	14.67	23.32	22.78	23.92	21.17	2.18
18	2.46	2.52	2.79	1.38	2.29	0.31
19	26.08	69.86	56.18	53.78	51.47	9.17
20	2.40	2.95	3.15	3.45	2.99	0.22
21	0.99	0.88	1.98	1.64	1.37	0.26
22	16.64	51.80	36.17	37.33	35.48	7.22
23	18.91	45.47	39.73	41.29	36.35	5.94
24	3.96	1.70	3.38	4.42	3.36	0.59
25	11.48	7.72	8.21	13.84	10.31	1.44
26	6.97	9.86	10.64	14.29	10.44	1.51
27	12.49	16.34	18.98	23.10	17.73	2.23

[a]See Table 50 for food additive culture medium concentrations.
[b]Results expressed as mean ± SEM of 4 replicate experiments (experiments A, B, C and D) in units of fold induction.

Table 53. Effect of treatment of human hepatocytes with individual food additives and food additive mixtures on CYP2B6 mRNA levels

Group[a]	Hepatocyte experiment				CYP2B6 mRNA[b]	
	A	B	C	D	Mean	± SEM
Control	1.00	1.00	1.00	1.00	1.00	-
1	1.41	1.63	1.32	2.47	1.71	0.26
2	1.50	2.81	3.33	1.99	2.41	0.41
3	2.19	3.25	3.09	2.85	2.85	0.23
4	4.09	4.25	7.04	7.08	5.62	0.84
5	6.98	7.17	8.24	11.96	8.59	1.16
6	7.14	14.14	20.04	23.75	16.27	3.63
7	0.67	1.53	1.14	0.86	1.05	0.19
8	0.75	1.85	1.81	1.09	1.37	0.27
9	0.33	2.30	1.81	0.85	1.32	0.45
10	0.44	1.44	1.01	0.53	0.86	0.23
11	1.21	1.69	1.36	1.05	1.33	0.14
12	1.20	1.86	1.71	1.39	1.54	0.15
13	4.13	4.86	5.86	6.37	5.30	0.50
14	1.71	2.58	3.31	4.37	2.99	0.56
15	1.08	2.42	1.62	2.30	1.86	0.31
16	1.82	4.00	3.62	2.36	2.95	0.51
17	2.37	4.23	5.39	2.03	3.51	0.79
18	0.87	1.60	1.23	0.92	1.16	0.17
19	5.00	14.12	14.85	9.18	10.79	2.30
20	1.36	3.61	5.59	3.28	3.46	0.87
21	1.32	4.25	5.00	3.58	3.54	0.80
22	3.89	10.29	9.91	8.12	8.05	1.47
23	2.77	8.04	8.60	9.47	7.22	1.51
24	1.19	1.60	2.23	2.84	1.97	0.36
25	1.19	2.12	2.51	2.26	2.02	0.29
26	0.91	3.91	3.08	2.98	2.72	0.64
27	1.81	6.16	8.39	5.55	5.48	1.37

[a]See Table 50 for food additive culture medium concentrations.
[b]Results expressed as mean ± SEM of 4 replicate experiments (experiments A, B, C and D) in units of fold induction.

Table 54. Effect of treatment of human hepatocytes with individual food additives and food additive mixtures on CYP3A4 mRNA levels

Group[a]	Hepatocyte experiment				CYP3A4 mRNA[b]	
	A	B	C	D	Mean	SEM
Control	1.00	1.00	1.00	1.00	1.00	-
1	1.41	1.27	0.98	1.36	1.25	0.10
2	1.65	2.65	1.73	1.58	1.90	0.25
3	2.27	2.59	3.04	1.80	2.42	0.26
4	1.77	1.94	1.84	1.46	1.75	0.10
5	1.73	1.62	1.70	2.86	1.98	0.30
6	3.87	3.48	3.47	3.98	3.70	0.13
7	0.96	1.65	1.29	1.05	1.24	0.15
8	1.04	1.87	1.76	1.39	1.52	0.19
9	0.63	1.82	1.35	0.97	1.19	0.26
10	0.82	1.10	0.94	1.07	0.98	0.06
11	1.38	1.60	0.91	0.96	1.21	0.17
12	1.42	1.41	1.59	1.28	1.42	0.06
13	2.87	2.45	1.97	2.02	2.33	0.21
14	2.06	2.84	3.46	1.90	2.57	0.36
15	1.42	1.48	2.10	1.49	1.62	0.16
16	1.45	2.20	1.35	1.24	1.56	0.22
17	1.73	1.08	1.33	1.31	1.36	0.13
18	0.77	2.11	1.55	0.71	1.29	0.34
19	1.43	2.54	3.60	2.70	2.57	0.44
20	1.47	3.75	2.98	2.00	2.55	0.51
21	1.33	3.37	2.87	1.72	2.32	0.48
22	1.83	2.50	1.95	1.42	1.92	0.22
23	0.96	1.81	1.74	1.54	1.51	0.19
24	1.18	1.26	1.44	1.24	1.28	0.06
25	0.70	1.68	1.14	0.92	1.11	0.21
26	0.53	1.52	1.22	1.07	1.09	0.21
27	0.93	2.22	2.28	1.16	1.65	0.35

[a]See Table 50 for food additive culture medium concentrations.
[b]Results expressed as mean ± SEM of 4 replicate experiments (experiments A, B, C and D) in units of fold induction.

Table 55. Effect of treatment of human hepatocytes with BNF, NaPB and RIF on CYP1A2, CYP2B6 and CYP3A4 mRNA levels

Treatment	CYP mRNA (fold induction)[a]		
	CYP1A2	CYP2B6	CYP3A4
Control	1.0	1.0	1.0
BNF 10 µM	101.1 ± 10.6***	n.d.[b]	n.d.
BNF 50 µM	286.0 ± 53.3***	n.d.	n.d.
NaPB 200 µM	n.d.	7.9 ± 2.3***	8.9 ± 0.8**
NaPB 1000 µM	n.d.	21.0 ± 5.8***	9.9 ± 3.8**
RIF 10 µM	n.d.	7.7 ± 0.6***	17.9 ± 3.5***
RIF 50 µM	n.d.	13.5 ± 3.8***	24.6 ± 3.5***

[a]Results are presented as mean ± SEM of 4 experiments.
[b]n.d., not determined.

Values significantly different from control are: **p<0.01; ***p<0.001

11. FIGURES

Figure 1. Effect of treatment of rats for 28 days with BHT and TB on hepatic CYP1A1 and CYP1A2 mRNA levels

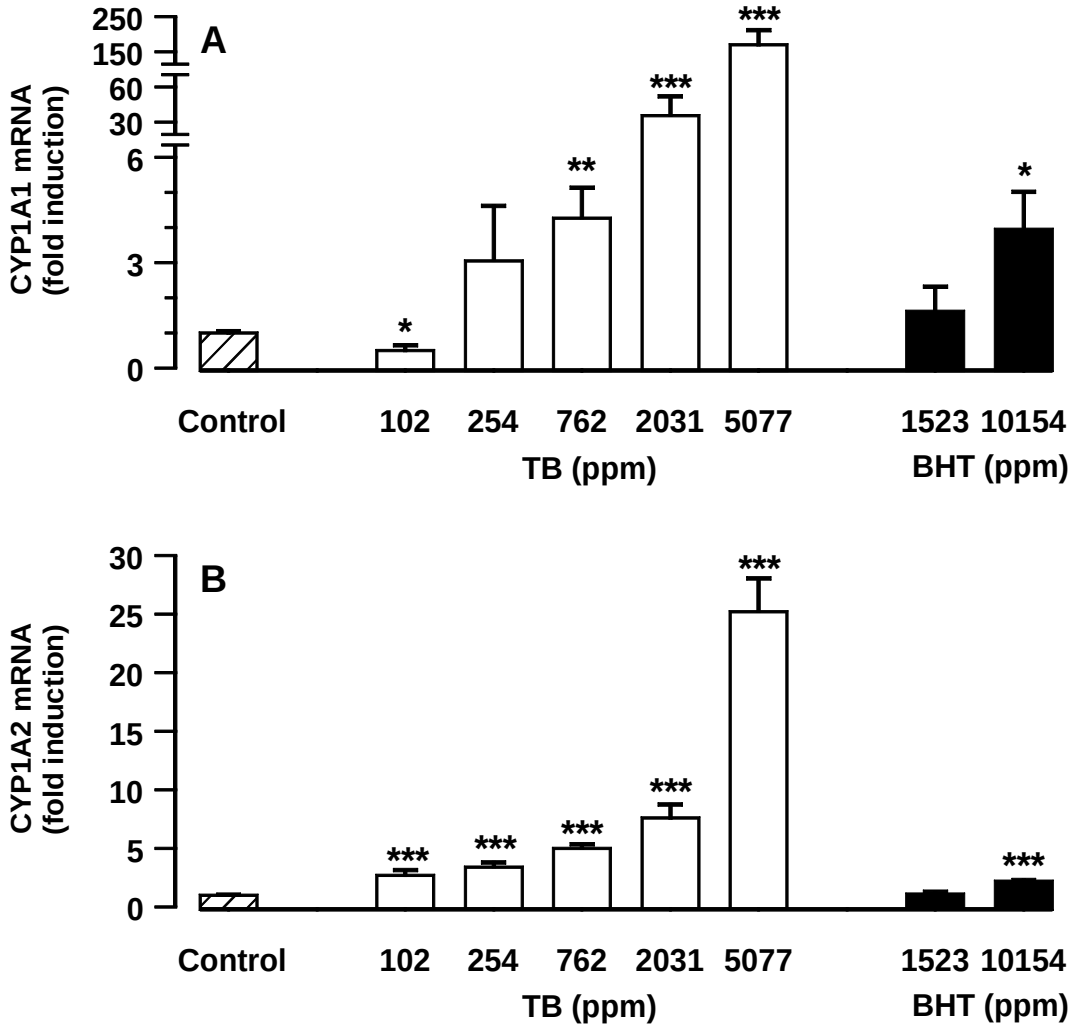

Fig. 1. Effect of feeding diets containing 102-5077 ppm TB and 1523 and 10154 ppm BHT for 28 days on levels of rat hepatic CYP1A1 (A) and CYP1A2 (B) mRNA. Total RNA was extracted from liver samples and levels of CYP form mRNAs determined by real-time quantitative RT-PCR methodology (TaqMan®). To normalise for RNA loading, levels of each CYP mRNA were expressed as a ratio to levels of albumin mRNA, which was co-amplified in a duplex reaction. Results are expressed as mean ± SEM for groups of 10 control and 6 TB and BHT treated rats. Values significantly different from control are: *p<0.05; **p<0.01; ***p<0.001.

Figure 8. Hierarchical clustering of gene expression profiles obtained from livers from rats exposed to individual compounds and mixtures of BHT, CC, PG and TB

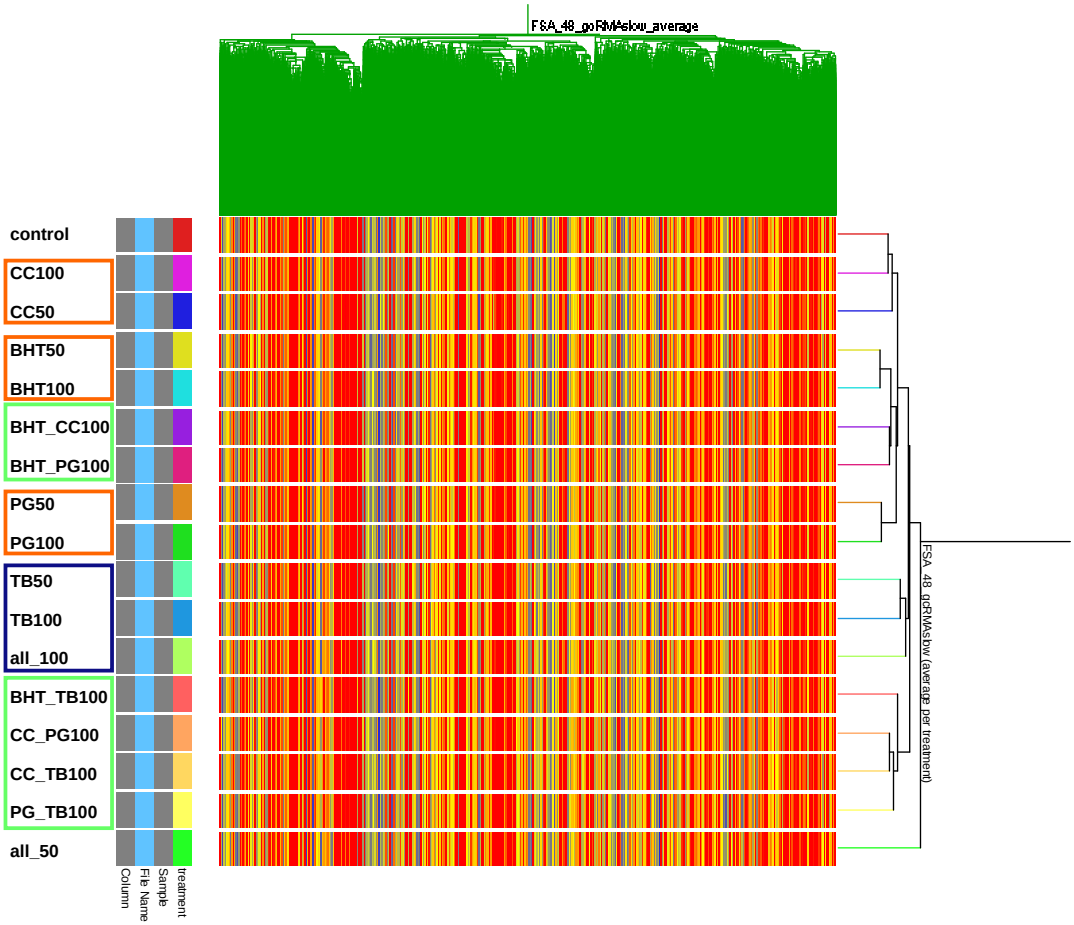

Figure 8. Hierarchical clustering (complete gene expression profile). Note the clustering of genes according to treatment: individual CC, BHT, PG, binary mixtures containing BHT, TB together with a quaternary mixture at highest dose levels, binary mixtures containing TB and quaternary mixture containing lowest dose levels

Figure 9. Principal component analysis of gene expression profiles obtained from livers from rats exposed to individual compounds and mixtures of BHT, CC, PG and TB

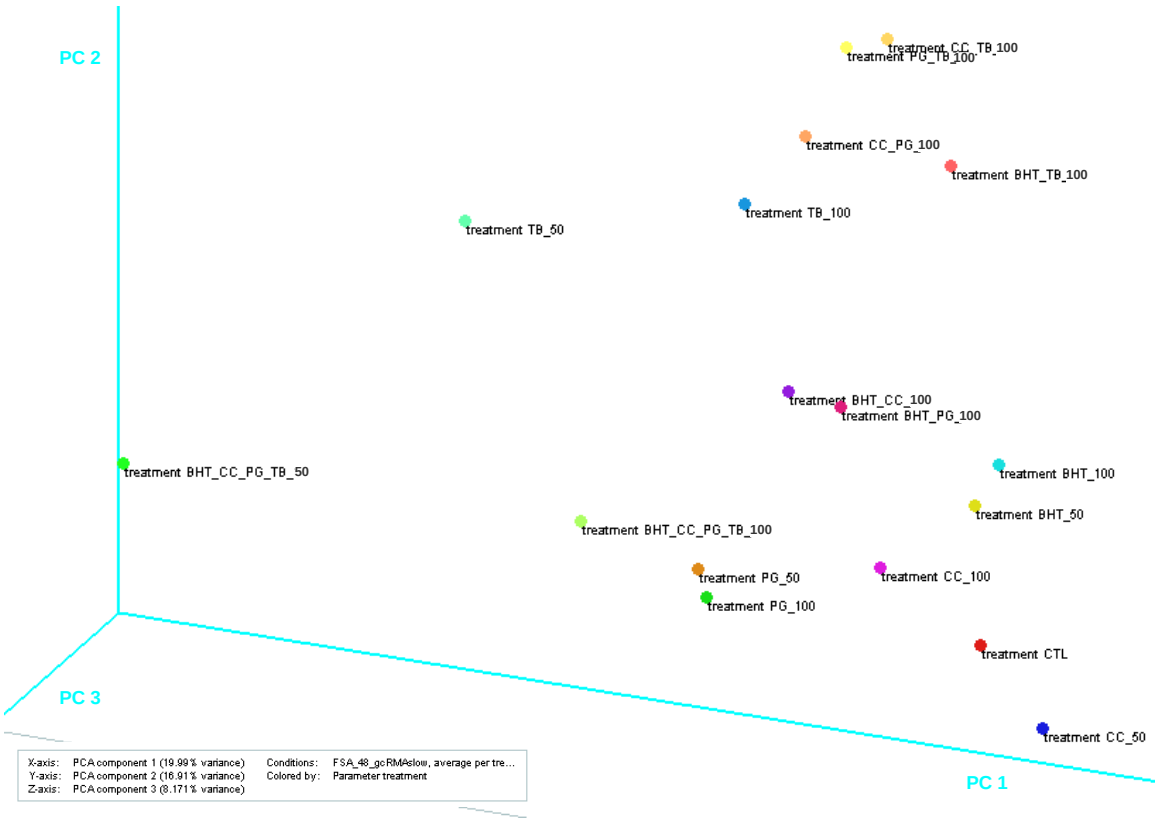

Fig.9. Principal component analysis (complete gene expression profile)

Figure 10. Number of differentially expressed genes compared to control, determined in livers from rats exposed to individual compounds and mixtures of BHT, CC, PG and TB

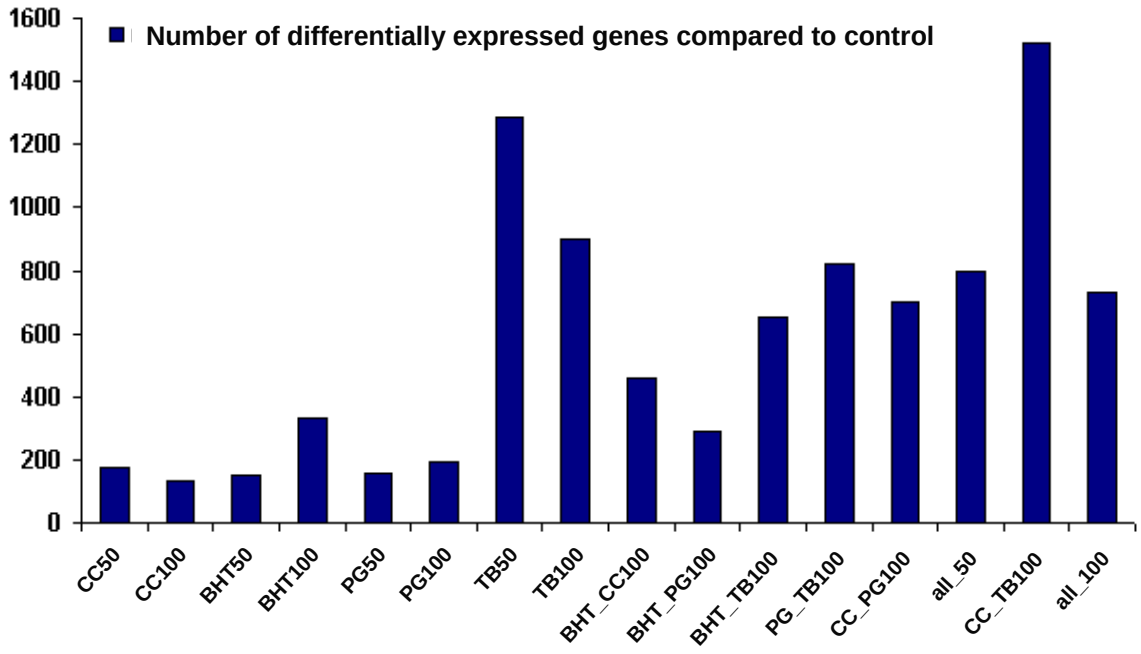

Fig 10. Number of differentially expressed genes between control group and each of the treatment groups (p-value <0.01, average absolute fold change greater than 1.5)

Figure 11. Heathmap of correlations between gene expression profiles, determined in livers from rats exposed to individual compounds and mixtures of BHT, CC, PG and TB

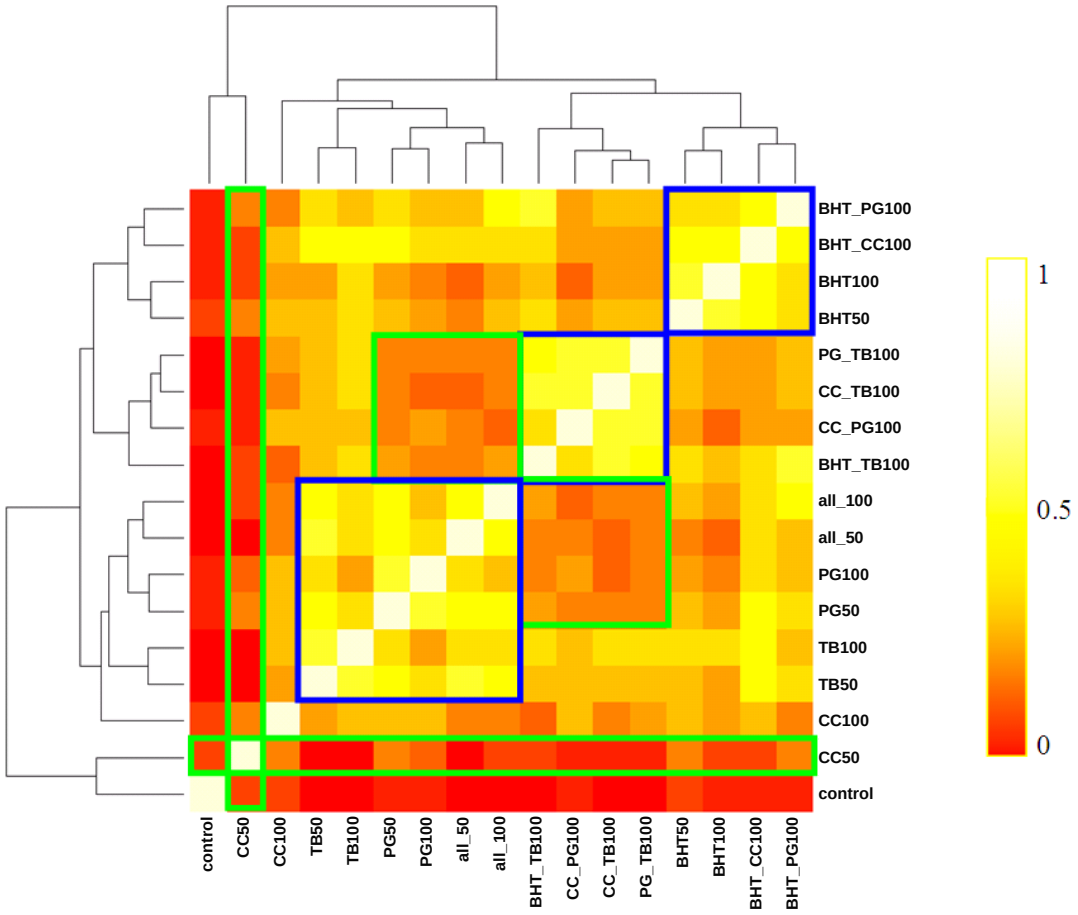

Fig. 11. Heatmap representing correlations between treatment effects. Person correlation for each pair of treatments was calculated using the $\log_2$ expression ratios (treatment versus control) of 3538 genes differentially expressed in any of the treatments. Color code: white – high correlation, red – low correlation

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Figure 12

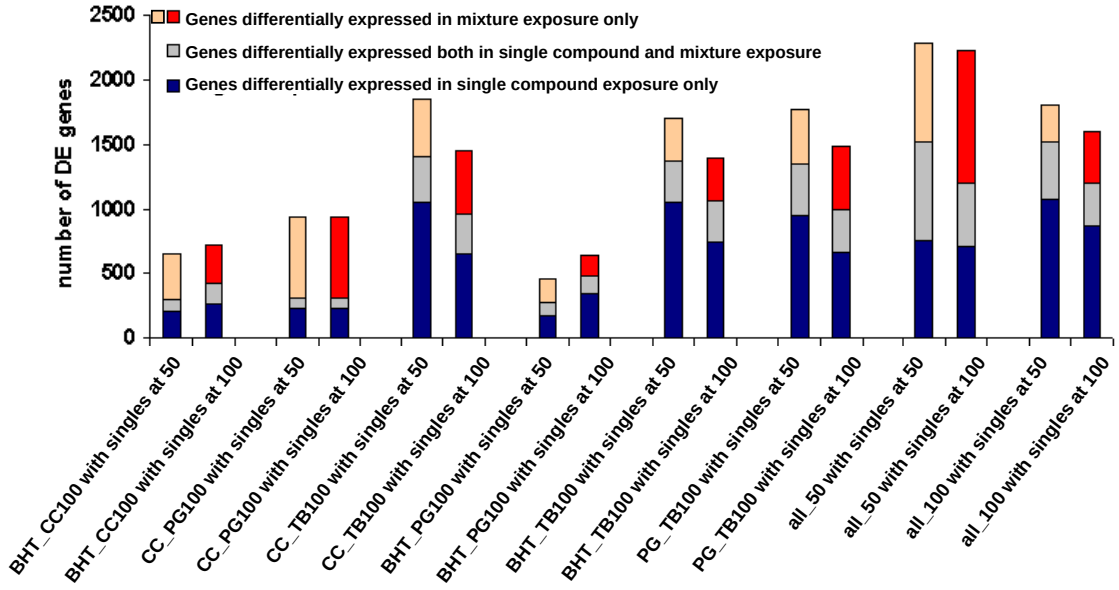

Fig. 12. Magnitude of mixture effects, based on number of differentially expressed genes, which can / can not be predicted from the combined effect of its individual components. Plotted are numbers of differentially expressed genes specific to mixture only (orange or red), specific for the individual compounds only (blue) and their overlap (gray). Marks “low” or “high” designate that the mixture effect was compared to the combined effect of single compounds in 50% (low) or 100% (high) fractional dosages.

Figure 13

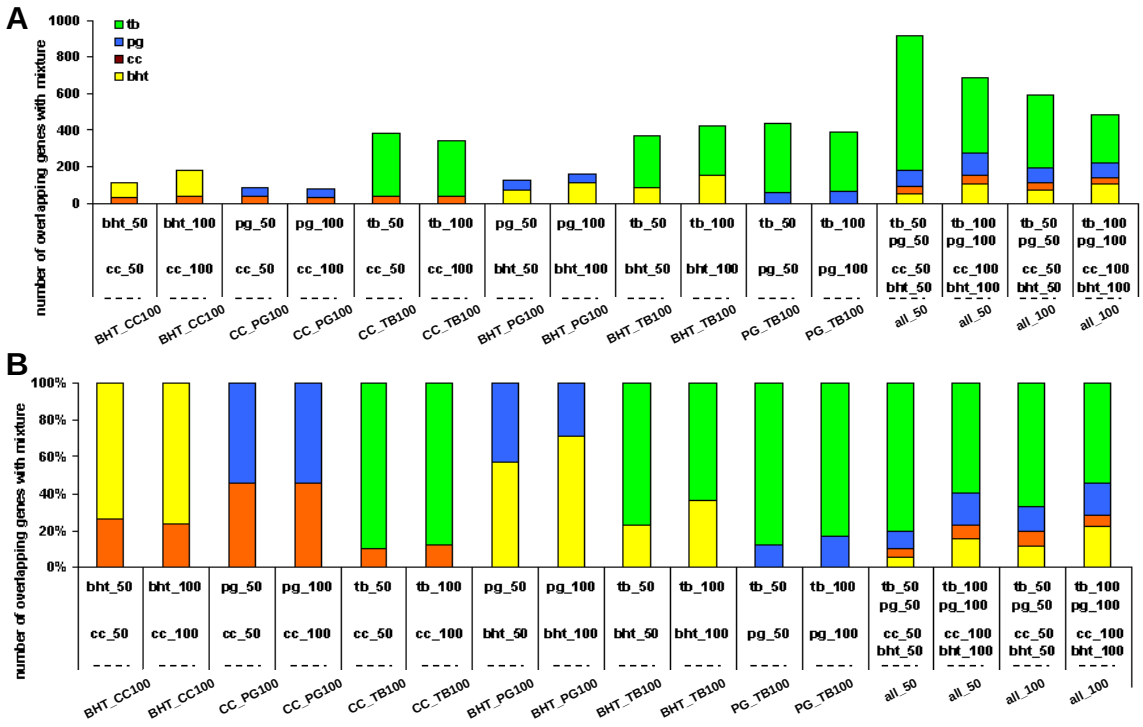

Fig.13A. Number of differentially expressed genes that are common to the mixture and each of the single components comprising the mixture. Bottom label represents the mixture (q-quaternary mixture) and two top labels represent single compounds and their doses. Bottom part of the bar corresponds to the value associated with the single compound with label at the lower position and the upper part of the bar corresponds to the value associated with the single compound with label at the top position. Relative size of each coloured part of the bar represents the contribution of the particular compound to the mixture effect. Color code: TB - green; PG - blue; CC - orange; BHT - yellow. Fig.13B. Contribution of the particular compound to the mixture effect expressed in percentages of the total overlap

Figure 20. Outcome of statistical analysis for inference of mixture effects in rat hepatocytes exposed *in vitro* to individual compounds and mixtures of BHT, CC, PG and TB: CYP1A2 mRNA

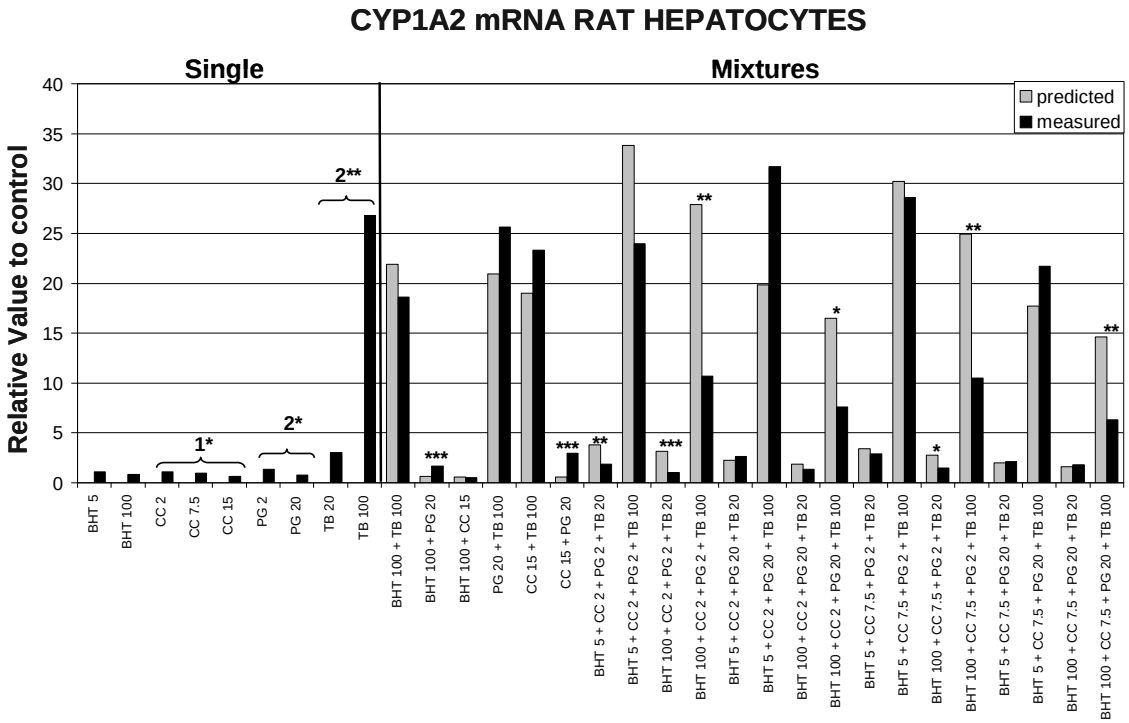

Fig 20. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on CYP1A2 mRNA levels in rat hepatocytes from Sprague Dawley rats exposed for 72 hours. Results are shown relatively to the observations in the control group. Left side represents the measured group averages in single compound exposures, relative to control values, together with statistical inference for dose dependencies: 1: linear term contributing to dose dependency of individual additive; 2: quadratic terms contributing to dose dependency of individual additive. Right side represents predicted and measured group averages for mixture exposures**.** Measured levels are the geometrical mean in comparison to control. Predicted levels in mixtures, in comparison to control, are based upon the following Additivity Surface Equation: $\ln(CYP1A2) = -0.002027*[BHT] - 0.0222*[CC] + 0.1666*[PG] - 0.008919*[PG]^2 + 0.06041*[TB] - 0.0002756*[TB]^2$, with concentrations in μM. *: $P < 0.05$; **: $P < 0.01$; *** $P < 0.001$ (Student t-test).

Figure 21. Outcome of statistical analysis for inference of mixture effects in rat hepatocytes exposed *in vitro* to individual compounds and mixtures of BHT, CC, PG and TB: CYP2B1 mRNA

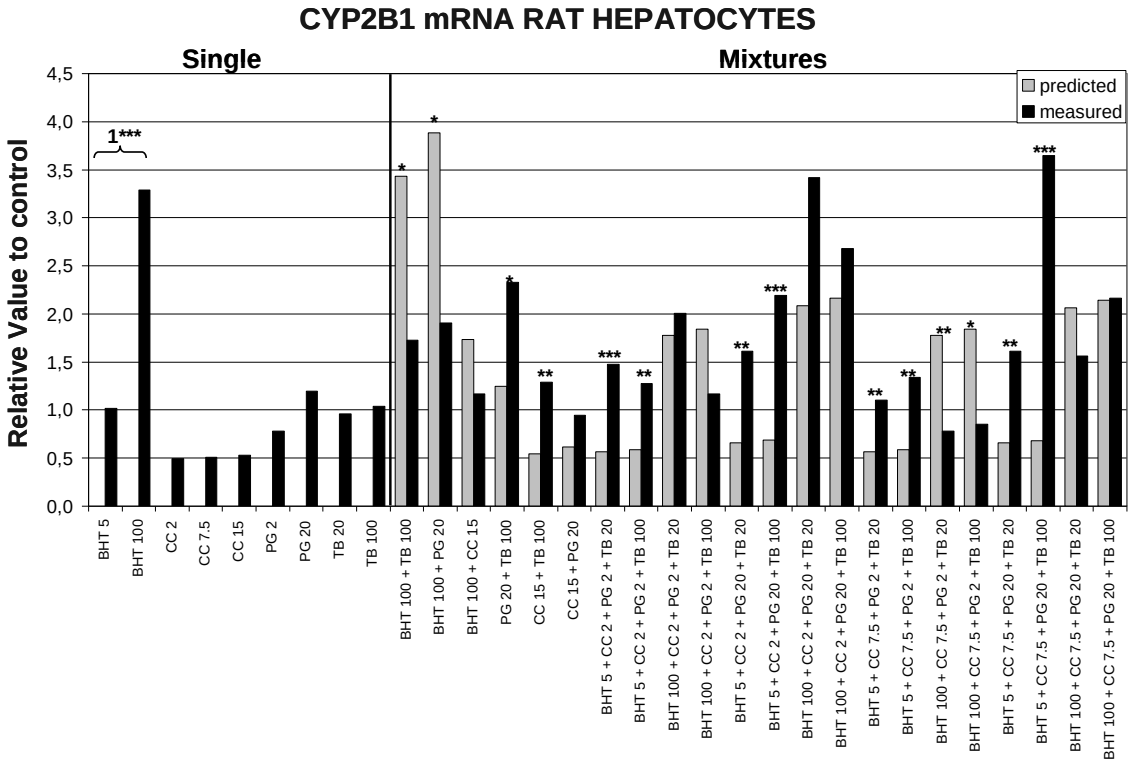

Fig 21. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on CYP2B1 mRNA levels in rat hepatocytes from Sprague Dawley rats exposed for 72 hours. Explanation is similar as in the legend of Fig. 20. 1: linear term contributing to dose dependency of individual additive. Additivity Surface Equation used to calculate the predicted values for mixtures: ln(CYP2B1)= 0.012*[BHT]-0.659# +0.00843*[PG]+0.000442*[TB], with concentrations in μM. #: indicator variable, present in equation if CC is present in mixture, absent in equation if CC is absent in mixture.

*: P < 0.05; **: P < 0.01; *** P < 0.001 (Student t-test).

Figure 22. Outcome of statistical analysis for inference of mixture effects in rat hepatocytes exposed *in vitro* to individual compounds and mixtures of BHT, CC, PG and TB: BFC O-debenzylase activity

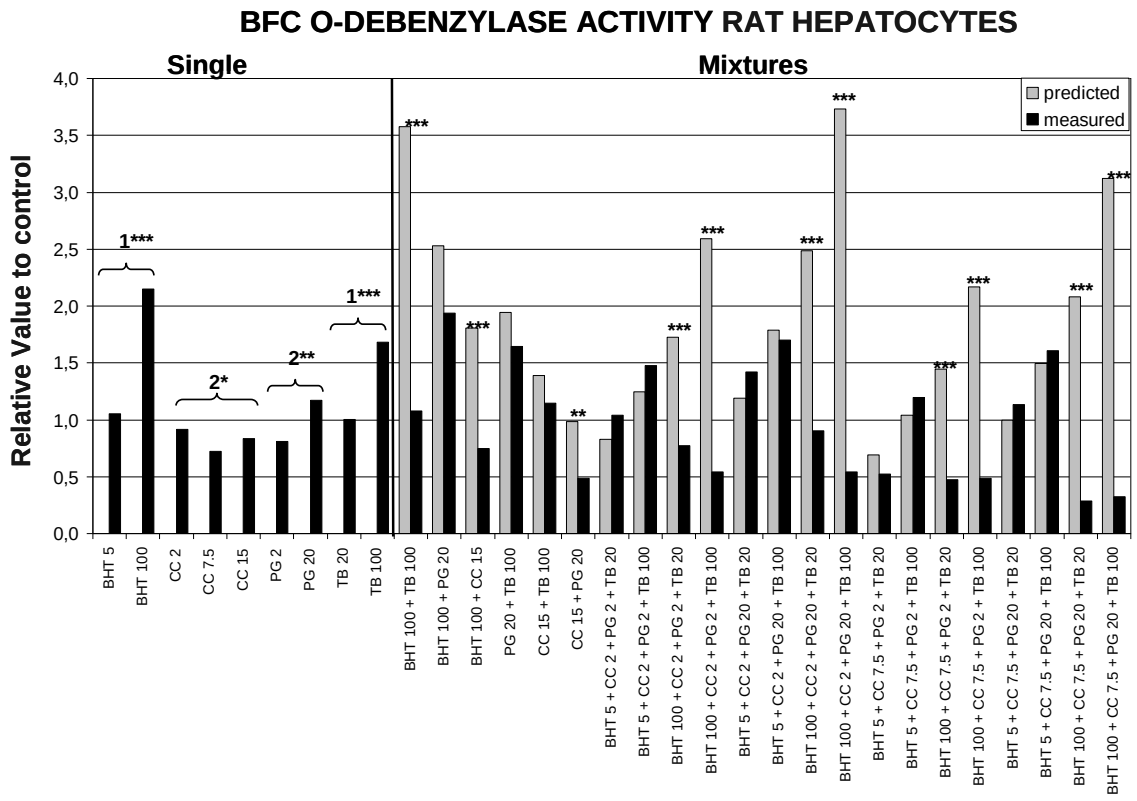

Fig. 22. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on BFC O-debenzylase activity levels in rat hepatocytes from Sprague Dawley rats exposed for 72 hours. Explanation is similar as in the legend of Fig. 20. 1: linear term contributing to dose dependency of individual additive; 2: quadratic terms contributing to dose dependency of individual additive.

Additivity Surface Equation used to calculate the predicted values for mixtures:

$\ln(BFC) = 0.00774*[BHT]-0.0682*[CC]+0.00377*[CC]^2-0.113*[PG]+0.00608*[PG]^2 +0.00508*[TB]$, with concentrations in µM. *: $P < 0.05$; **: $P < 0.01$; *** $P < 0.001$ (Student t-test).

Figure 23. Outcome of statistical analysis for inference of mixture effects in human hepatocytes exposed *in vitro* to individual compounds and mixtures of BHT, CC, PG and TB: CYP1A2 mRNA

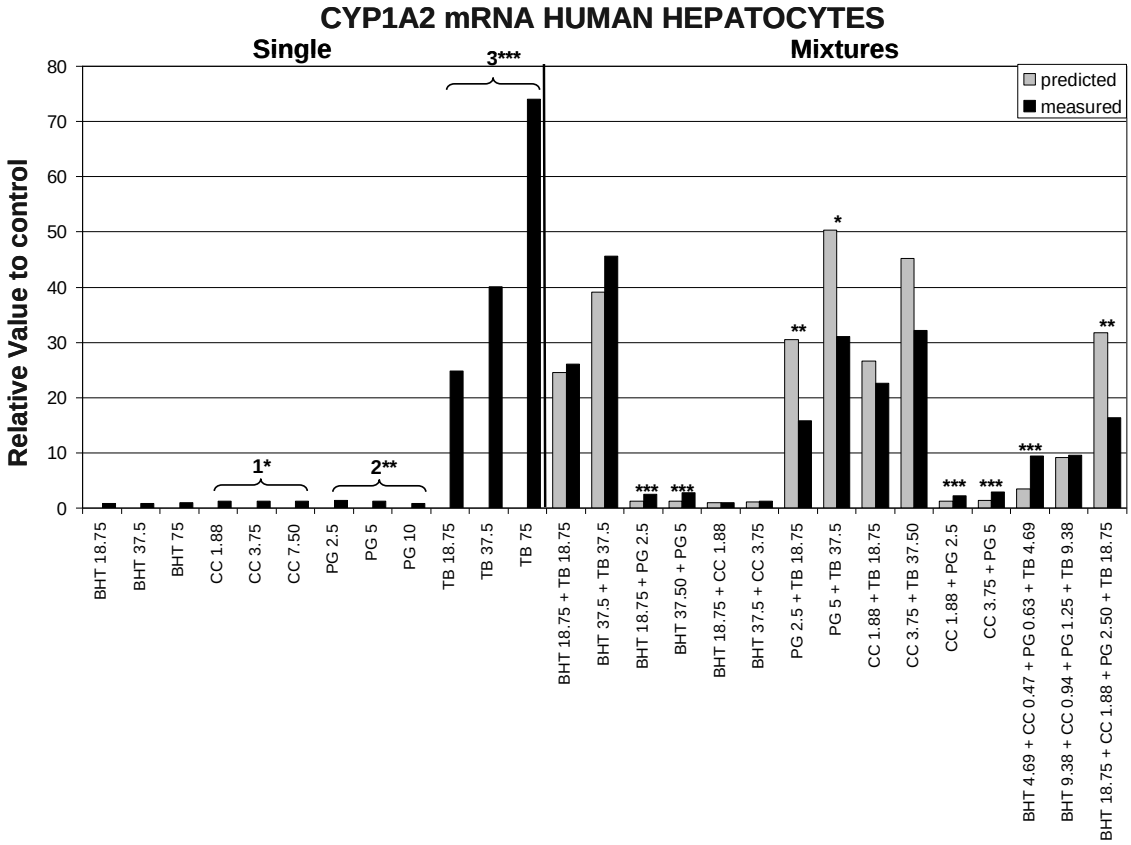

Fig. 23. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on CYP1A2 mRNA levels in hepatocytes from human donors exposed for 72 hours. Explanation is similar as in the legend of Fig. 20. 1: linear term contributing to dose dependency of individual additive; 2: quadratic terms contributing to dose dependency of individual additive. 3: cubic term contributing to dose dependency of individual additive. Additivity Surface Equation used to calculate the predicted values for mixtures: $\ln(CYP1A2) = -0.000705735*[BHT]+0.0313*[CC]+0.113*[PG]-0.0136*[PG]^2 +0.280*[TB]-0.00670*[TB]^2+4.98453^{-5}*[TB]^3$, with concentrations in μM. *: $P < 0.05$; **: $P < 0.01$; *** $P < 0.001$ (Student t-test).

Figure 24. Outcome of statistical analysis for inference of mixture effects in human hepatocytes exposed *in vitro* to individual compounds and mixtures of BHT, CC, PG and TB: CYP2B6 mRNA

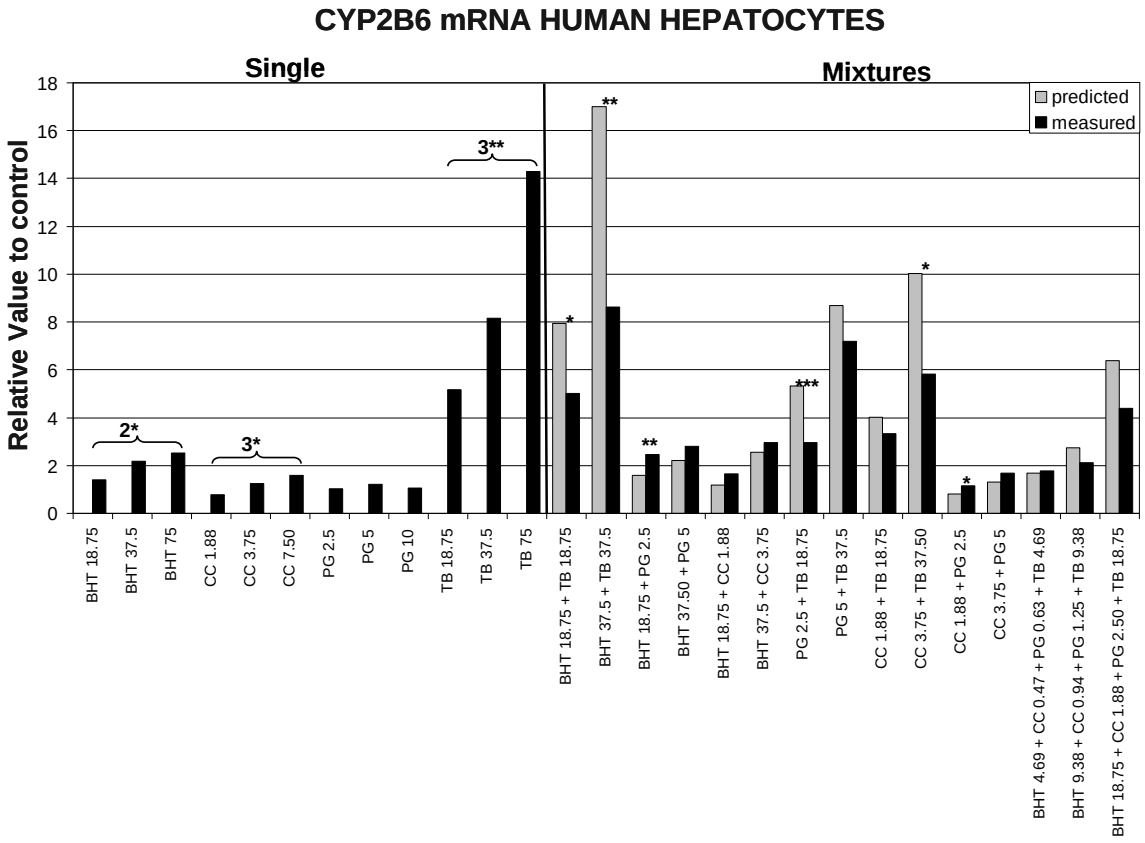

Fig. 24. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on CYP2B6 mRNA levels in hepatocytes from human donors exposed for 72 hours. Explanation is similar as in the legend of Fig. 20. 1: linear term contributing to dose dependency of individual additive; 2: quadratic terms contributing to dose dependency of individual additive. 3: cubic term contributing to dose dependency of individual additive. Additivity Surface Equation used to calculate the predicted values for mixtures: $\ln(CYP2B6)=0.0265*[BHT]-0.000186*[BHT]^2-0.4478*[CC] +0.2003*[CC]^2-0.0177*[CC]^3+0.0125*[PG] +0.1333[TB]-0.00282*[TB]^2+2.02^{-5}[TB]^3$, with concentrations in μM. *: $P < 0.05$; **: $P < 0.01$; *** $P < 0.001$ (Student t-test).

Figure 25. Outcome of statistical analysis for inference of mixture effects in human hepatocytes exposed in vitro to individual compounds and mixtures of BHT, CC, PG and TB: CYP and TB: CYP3A4 mRNA

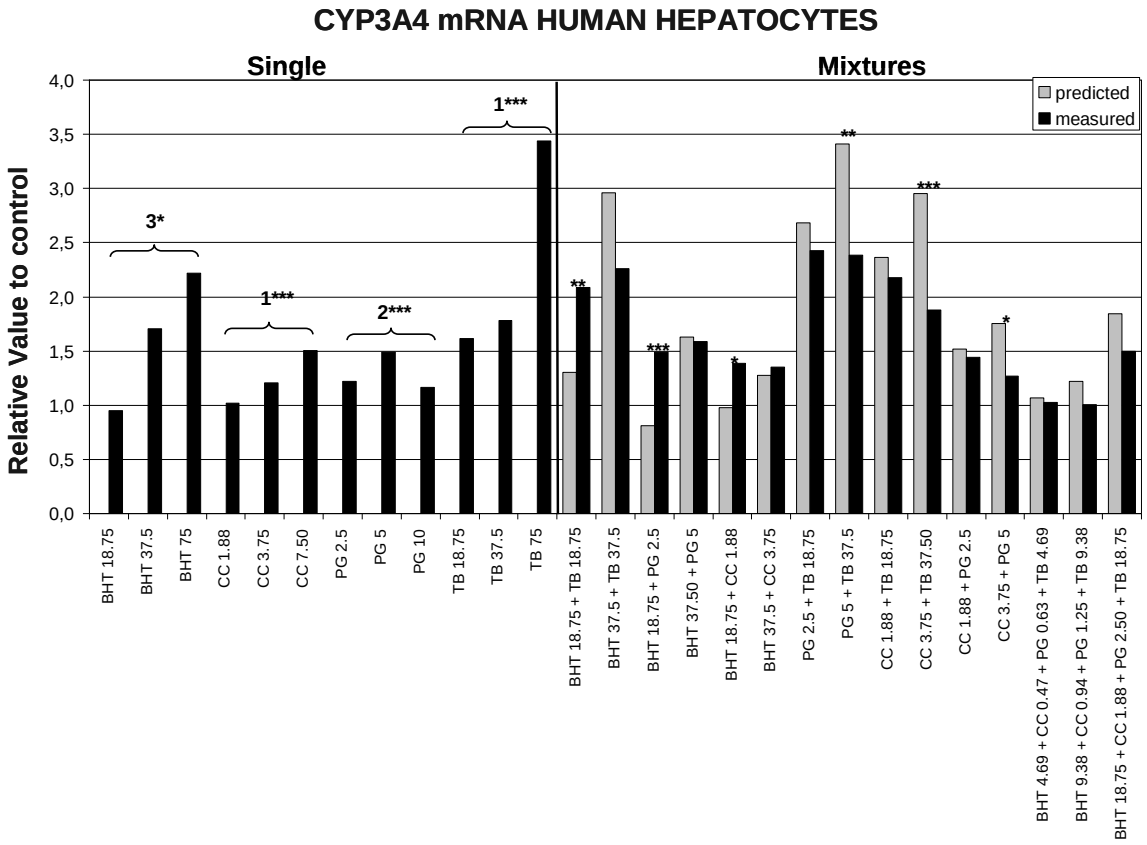

Fig. 25. Effects of single compounds and mixtures of food additives BHT, CC, PG and TB on CYP3A4 mRNA levels in hepatocytes from human donors exposed for 72 hours. Explanation is similar as in the legend of Fig. 20. 1: linear term contributing to dose dependency of individual additive; 2: quadratic terms contributing to dose dependency of individual additive. 3: cubic term contributing to dose dependency of individual additive. Additivity Surface Equation used to calculate the predicted values for mixtures: $\ln(CYP3A4) = -0.0322*[BHT] + 0.0019*[BHT]^2 + -1.77^{-5}*[BHT]^3 + 0.0519*[CC] + 0.1303*[PG] - 0.0114*[PG]^2 + 0.0166[TB]$, with concentrations in μM. *: $P < 0.05$; **: $P < 0.01$; *** $P < 0.001$ (Student t-test).

Supplementary statistics to Project Development of methods for the assessment of the health effects from mixtures of food additives

Supplementary outline on statistical analysis ('s'), tables, results and guidance to suplementary data files ('df')

1. Outline of statistical analysis

The analysis described here involves a detailed statistical analysis on mixture effects of clinical and biochemical parameters from a 28 day *in vivo* combination study with selected food additives BHT, CC, PG and TB.

2. Purpose of the analysis

Purpose of the statistical analysis outlined here was to address the question whether the effects on parameters above of administration of primarily binary but also quaternary mixtures of the food additives BHT, CC, PG and TB can possibly be categorized according to any of the three basic concepts in mixture toxicology. These concepts which are at the base of the statistical analysis outlined below include (1) independent (or dissimilar) joint action, also known as response addition, (2) similar joint action, also known as dose addition and (3) interaction:

- Response or effect addition (although slightly different see Table on page 3), these terms are often used interchangeably), is a type of joint action which is non-interactive, namely, each chemical in the mixture does not affect another's toxicity. The *modes of action* and possibly the nature and site of toxic effect *differ* among the chemicals in the mixture. The toxicity of the mixture can be predicted (calculated) from the dose-response curves obtained from the individual chemicals.
- Similar joint action or dose addition is also non-interactive. The chemicals produce similar but independent effects, so that one chemical can be substituted at a constant proportion of the other. The *mode of action* of chemicals in case is believed to be *similar*. The toxicity of the mixture can be predicted (calculated) using a summation of the doses of each individual chemical after adjusting for the differences in potencies. The adding of doses implies that the summed dose can be high enough to induce a toxic

effect, even when the dose of each individual chemical is at a level below its individual effect threshold. One should realize that the above theoretical distinction between dose and response addition generally does not hold so strictly in whole organisms, because of the complexity of physiological systems

- Interaction is characterized by one chemical influencing the biological action of another. Interaction can be defined as the type of joint action showing a mixture response that deviates from that expected from response or dose addition. Interaction can be less that additive (antagonistic) or greater than additive (synergistic).

Therefore, the statistical analyses outlined below aim at testing whether the outcome of parameters actually measured for binary and quaternary mixtures deviates from those predicted by equations modeling response and dose additions based on the measurements obtained from individual (and binary mixtures) of additives. To illustrate the analyses, the outcome of the statistical analyses is primarily discussed in detail in sections below for body weight as an example. All raw data pertaining to the statistical analysis are contained in the Supplementary electronic files: 'DF1_Data Summary'; 'DF2_t tests.out'; 'DF3_additivitysurf.out'; 'DF4_nonadditivity.out'; 'DF5_orderednonadditivity' and 'DF6_interaction.out'.

3. Dataset used for analysis

A summary of all data obtained and used in the present statistical analysis is shown in supplementary electronic file 'DF1_Data Summary'. This file includes 31 columns. The first 11 of these describe details of the experimental conditions. These include a group number, an animal number, a description of the treatment applied in the group, nominal values of BHT, CC, PG, and TB, respectively, and measured values of the contents on these compounds. The 20 columns following the details of the experimental conditions are body weight, total liver weight, relative liver weight, cytochrome P450 (CYP) content, activity of 7PR, activity of 7ER, GST activity measured through CDNB, GST activity through DCNB, and induction of RNA for CYP2B1 and CYP1A2, respectively. CYP content and all the activities were included in three versions, plain or specific activity, activity per gram of liver, and activity per relative liver weight. A total of 174 animals were included in the study. For body weight, liver weight, relative liver weight, CYP, and all the enzyme activities determined, results were recorded for all the animals. However, for three control animals, and for animal number 51 from group 8 (762 ppm), there were problems with the RNA isolation. Accordingly, the CYP1A2 and CYP2B1 mRNA data from these animals were not included.

4. Calculations of fractional dosages and annotation of groups

To facilitate comparisons of effect-sizes for the various compounds, fractional dosages were calculated through dividing the nominal or actual dosage by the maximum dosage.

Two examples are given:

- BHT low dose group:
    - Fractional dosage: BHT nominal dosage of 761.5 ppm/BHT maximal dosage of 3046 ppm = 0.25
- Binary mixture of CC nominal dosage of 1015.5 ppm and TB nominal dosage of 437.5 ppm
    - Fractional dosage of CC in mixture: CC nominal dosage of 1015.5 ppm/CC maximal dosage of 4062 ppm = 0.25
    - and fractional dosage of TB in mixture: TB nominal dosage of 437.5 ppm/TB maximal dosage of 1750 ppm = 0.25

For the nominal dosages, fractional values of 0.0625, 0.125, 0.25, 0.5 and 1 where obtained. For the fractional dosages calculated from the actual dosages the maximum value is still 1, while the other values are near those just listed (based on difference between nominal and actual dosage).

Subsequently, the dosage groups were labelled according to the compounds administered in the food and the dose obtained by adding the coded values of the fractional nominal dosage expressed as percentage. For example, the group labelled 'CC_TB50' has CC administered at 25% of the maximum value of 4062 ppm and TB administered at 25% of the maximum value of 1750 ppm. Whereas for labelling of groups fractional nominal dosages were used, calculations on the effect addition and dose addition were based on the fractional dosages calculated from the actual dosages employed throughout the study.

5. Checks on outliers; t tests against the control and determination of the effects of data transformation on normality and homogeneity on data.

For all the parameters, four graphical checks were performed to detect outliers or gross departures from normality and homogeneity of the variation between the animals. The basic ingredients of these plots were the 174 differences between the parameter-value from a particular animal with the mean value for the experimental group to which the animal belongs. These values, called *residuals*, were standardized by division through their standard error. The checks (plots not shown) were:

- A histogram of the standardized residuals. An ideal histogram would be bell shaped with most values between –2 and +2. Outliers would be in the left or right tails of the histogram.
- A plot of the standardized residuals ordered from small to large against theoretical values derived from a standard normal distribution. An ideal normal plot would show a straight line through the origin, with slope +1.
- A half-normal plot. This plot is based on absolute values of the standardized residuals rather than the original values. Again, an ideal half-normal plot would show a straight line through the origin with slope +1.
- A plot of residuals against the group means. This plot, called a fitted value plot, checks for homogeneity of the variation between the animals.

For each parameter, two sets of four plots were made. One was based on the original values. The other set was based on the natural logarithm of the original values. The untransformed activity measurements and the untransformed mRNA data were incompatible with assumptions of normality and homogeneity of variation, while the natural log-transformed values were compatible with these assumptions. For CYP, relative and total liver weights, and body weight, both untransformed and transformed data were compatible with the above assumptions. It was decided to use natural log-transformed values in all cases. Supplementary Table S-1 lists parameters with outlier values for some of the animals. These values were removed in all subsequent analyses.

Table S-1. Outliers in the data

Parameter	**Group**	**Animal**
body weight (g)	20 (all 100)	125
	23 (BHT_TB 50)	141
total liver weight (g)	10 (PG 100)	66
	20 (all 100)	125
Relative liver weight (g/100 g body weight)	10 (PG 100)	66
CYP (nmol/mg)	16 (BHT TB 100)	98
CYP (nmol/g liver)	16 (BHT TB 100)	98

For all 20 parameters, an F test was employed to assess whether the 28 experimental groups differ with respect to each of the parameters. The F values are to be considered as signal to noise ratios. Supplementary Table S-2 gives a summary. As a quantification of the noise, the coefficient of variation of the data was included. These could be quite high. The largest value,

the one for CYP2B1, is 91%. In spite of the high between-animal variation, there were statistically significant differences between the experimental groups for all the 20 parameters considered here. In all cases, P values of less than 0.001 were obtained. It was surprising to see the largest signal to noise ratio (41.16) for the parameter with the largest coefficient of variation (CYP2B1).

Table S-2. F tests for 20 parameters

Parameter	**F**	**cv (%)**
body weight (g)	3.25	5.9
total liver weight (g)	3.3	10.3
Relative liver weight (g/100 g body weight)	5.76	7.1
CYP (nmol/mg)	3.1	9.2
CYP (nmol/g liver)	8.56	12.4
CYP (nmol/liver weight/kg body weight)	14.7	13.3
7PR (pmol/minute/mg)	99.8	32.5
7PR (nmol/minute/g liver)	99.3	34.2
7PR (nmol/minute/relative liver weight)	107	34.4
7ER (pmol/minute/mg)	10.6	24.2
7ER (nmol/minute/g liver)	13.5	25.9
7ER (nmol/minute/relative liver weight)	14.9	27.2
CDNB (µmol/minute/mg)	10.88	22.8
CDNB (µmol/minute/g liver)	8.09	24.6
CDNB (µmol/minute/relative liver weight)	12.63	23.9
DCNB (nmol/minute/mg)	14.13	15.8
DCNB (µmol/minute/g liver)	10.36	16.6
DCNB (µmol/min/relative liver weight)	19.33	15.5
CYP2B1 (fold induction)	41.16	91.2
CYP1A2 (fold induction)	18.09	57.4

Subsequent to the F tests, the difference between each of the treatment groups with the control was determined by means of t tests. The results for F tests and t tests are given in a separate Supplementary Electronic File called ‘DF2_t tests.out’. As a tool for preliminary study of dose dependencies a list of group means ordered from low to high values is included in this file for

each parameter. Some of the results are highly suggestive for the activity of one or two particular compounds and are summarized below:

- Relative liver weight: highest 8 means ordered according to BHT dose.
- CYP: highest 5 means ordered according to BHT dose.
- 7PR: highest 10 means ordered according to BHT dose.
- 7ER: highest 6 means ordered according to TB dose (less clearly so when related to RLW).
- CYP2B1: highest 10 means ordered according to BHT dose.
- CYP1A2: TB-ordered set of highest 3 means.

Note that these observations do not imply that the compounds not highlighted here do not have an effect. The assessment of the effects of the compounds will be discussed next.

6. Assessment of the effect additivity surfaces

A central issue in this study is to test the hypothesis of effect-addition. Therefore, equations describing the dose-dependency of each parameter in the single-exposure groups were calculated and values predicted for binary mixtures from these equations were compared to values found for the respective parameter in the combined exposure groups. For each parameter, the equation is said to model the additivity surface. Deviation from the additivity surface implicates that the mixture under study is not compatible with response addition. The additivity surface was established as follows. First, it was tested whether an equation with linear and quadratic terms for all 4 compounds would fit the data well. This appears to be the case with all parameters except for the three 7PR-related parameters and CYP2B1. Plots were generated for the fitted quadratic equations against each of the compounds (fractional actual value). For the parameters whose dose-dependencies are well described by the quadratic equation, non-significant terms ($P > 0.10$) were removed from the equation. Terms with P-values between 0.05 and 0.10 were not removed, to make sure that the equation fits the single dosage groups well. For 7PR, the plot of the fitted and observed relationship with the compounds pointed to an aberrant behaviour of the 25% CC group: the value was 1.489 ± 0.144 higher than in the remaining dosage groups. There was no other dose-dependency on CC for this parameter. For CYP2B1, a cubic term to describe the influence of CC satisfactorily was needed. There is a marked rise from control to CC25, followed by a gradual decline. Though less markedly, there was also an aberrant behaviour of the PG50 group. The value was 1.034 ± 0.373 higher than in the remaining groups. There was no

further dose-dependency on PG for this parameter. The results for the additivity surface are in Supplementary Electronic File 'DF3_additivitysurf.out'. This file contains the coefficients of the equation, and an overview of the assessment of the surface. The surfaces for CYP2B1 and 7PR taking into account the abberant behaviour of CC25 and PG50 are at the end of this file. As an example, the output for body weight is discussed. The table of coefficients (supplementary Table S-3) follows.

Table S-3.

```
***** Regression Analysis *****

 Response variate: BW
     Fitted terms: Constant, BHT, CC, PG, TB, BHT2, TB2

*** Estimates of parameters ***

                  estimate         s.e.     t(77)  t pr.
Constant            5.6990       0.0128    445.53  <.001
BHT                -0.1970       0.0944     -2.09  0.040
CC                 -0.0156       0.0271     -0.58  0.565
PG                 -0.0446       0.0267     -1.67  0.099
TB                  0.0181       0.0925      0.20  0.845
BHT2                0.1779       0.0960      1.85  0.068
TB2                -0.1718       0.0946     -1.82  0.073
```

Apparently, the assessment resulted in an equation that is quadratic for BHT and TB, and linear for CC and PG. The equation is:

$$\ln BW = 5.699 - 0.197\ BHT + 0.1779\ BHT^2 - 0.0156\ CC - 0.0446\ PG + 0.0181\ TB - 0.1718\ TB^2$$

The effect of CC is tiny and non-significant. The linear effect of PG and the quadratic effects of BHT and TB are of borderline significance: $0.05 < P < 0.10$. For all additive surface equations, all effects of borderline significance are retained to make sure that the combined equation fits the data well. Thus, non-additivity in the combined exposures cannot be attributed to a badly fitting additivity surface. A summary of the assessment of the surface for body weight is outlined below. This is the analysis of variance table for ln (body weight) (Table S-4):

Table S-4.

```
*** Accumulated analysis of variance ***

Change              d.f.          s.s.          m.s.       v.r.  F pr.
+ BHT
+ CC
+ PG
+ TB                   4     0.095019     0.023755       6.55  <.001
+ BHT2
+ CC2
+ PG2
+ TB2                  4     0.027498     0.006874       1.89  0.121
- PG2                 -1     0.000000     0.000000       0.00  0.992
- CC2                 -1    -0.000307     0.000307       0.08  0.772
+ Trt                  6     0.008275     0.001379       0.38  0.889
```

Residual	71	0.257582	0.003628
Total	83	0.388065	0.004675

The table shows that adding linear terms for all 4 compounds has a signal to noise ratio, or F value, of 6.55. Addition of quadratic terms has a smaller ratio of 1.89. The quadratic terms of PG and CC were removed subsequently.

To check whether it was forgotten to account for anything left in the data the F-value for adding the 4 cubic effects and the two quadratic effects previously removed was calculated. This gives the test in the line headed 'Trt'. The signal to noise ratio of 0.38 is not significant. Thus, it is confident that the additivity surface quantified in the table of coefficients (Table S-4) fits the data well. For 7PR, an equation was fitted with a so-called indicator variable to point to the CC25 group. For CYP2B1 an indicator variable for PG50 and a cubic term for CC were included.

A summary of the shape of the dose response curves is given in Table S-5, under the columns annotated as BHT, CC, PG and TB

Table S-5. Effects of the compounds in single exposure groups, in terms of dose dependency and overall tests on addition in the combined exposure groups

Parameter	BHT	CC	PG	TB	non-additivity
body weight (g)	2o	1ns	1o	2o	***
total liver weight (g)	1***	1ns	1ns	2*	ns
Relative liver weight (g/100 g body weight)	1***	1ns	1ns	1ns	ns
CYP (nmol/mg)	1***	1o	1ns	2o	ns
CYP (nmol/g liver)	1***	1ns	1o	2**	o
CYP (nmol/liver weight/kg body weight)	1***	1ns	1ns	2**	*
7PR (pmol/minute/mg)	2***	Cc25***	2**	1***	***
7PR (nmol/minute/g liver)	2***	Cc25***	2**	1***	***
7PR (nmol/minute/relative liver weight)	2***	Cc25***	2**	1***	***
7ER (pmol/minute/mg)	1***	1ns	2o	1***	Ns
7ER (nmol/minute/g liver)	1***	1ns	2*	2o	Ns
7ER (nmol/minute/relative liver weight)	1***	1ns	2*	2*	Ns
CDNB (µmol/minute/mg)	1***	1ns	1ns	1***	**
CDNB (µmol/minute/g liver)	1***	1ns	1ns	1***	Ns
CDNB (µmol/minute/relative liver weight)	2*	1ns	1ns	2o	Ns
DCNB (nmol/minute/mg)	1***	1ns	1*	2*	***
DCNB (µmol/minute/g liver)	1***	1ns	1ns	1***	***
DCNB (µmol/min/relative liver weight)	2*	1ns	1ns	2*	***
CYP2B1 (fold induction)	2***	3***	pg50**	2*	***
CYP1A2 (fold induction)	1ns	1o	2**	2***	***

ns: P>0.10; o: P < 0.10; *: P < 0.05; **: P < 0.01; *** P < 0.001

1: indicated linear term contribution to dose response curve

2: indicates quadratic term contributing to dose response curve

3: indicates cubic term contributing to dose response curve

7. Testing of deviation from effect addition in the combined exposure groups

For each of the 15 combined exposure groups and for each of the parameters the difference between the actual mean of the group and the value predicted by the additivity surface was

calculated. A t tests was employed to assess whether the difference was compatible with the hypothesis of effect addition. The results are in the Supplementary Electronic File 'DF4_nonadditivity.out'. Further, Tables S-10 through S-16 at the end of this document contain all these data as well in the format of measured group average +/- SD, the geometrical mean being *e* raised to the power of the average of all natural logarithms of the individual animal values, and the predicted value. Results on an overall test on additivity are included in Table S-5 (right column). The differences between the actual mean of the group and the value predicted by the additivity surface were also put in ascending order to permit the study of patterns; see Supplementary Electronic File 'DF5_orderednonadditivity'. The table in this file has the additional feature of expressing the difference on log-scale as a multiplication factor for the original scale. For example, the mean ln BW has for the all 100 group a value that is 0.1048 higher than predicted on the basis of effect addition. The difference is statistically significant (P = 0.004). The value of 0.1048 corresponds with a multiplication factor of 1.11.

To deal with the strange effect of CC on 7PR two approaches were tried. First, it was considered as if all groups with a nominal fractional value of 0.25 would have their 7PR raised with 1.489 (so called indicator variable). Then it was observed that CC_PG50, all100, and CC_TB50 have differences between actual and predicted values of *minus* this value. This suggests that there could be something wrong with the CC25 7PR measurements. In a second approach to deal with the effect of CC on 7PR, it was hypothesized that CC was actually inactive, and the results of CC25 were effectively disregarded. This made the 'non-additivities' for the above three groups disappear. However, the BHT_CC50 group now has a value 0.475 above the value under addition. So one way or the other CC seems to do something, but the effect is not clear. Both the above approaches were also tried for the effect of PG on CYP2B1. The non-additivity becomes more pronounced if the results of PG50 are disregarded.

Taken together, whenever the outcome for overall tests on additivity in the combined exposure groups is considered deviation from additivity was observed for all parameters tested, except for liver weight, relative liver weight and 7ER

8. More detailed assessment of deviation from effect addition: assessment of possible dose dependent interactions.

Up to this point, whenever applicable, the data on activities were analyzed in three versions: plain or specific activities expressed as activity per minute per mg protein, activities related to liver weight; and activities related to relative liver weight. From this point onward the attention is restricted to the plain activities. Table S-6 presents a detailed assessment of non-additivity as detected earlier, more in detail for binary mixtures. An explanation of the various columns follows.

Table S-6. Characterisation of non-additivity in binary and quaternary exposure groups

	non-additivity		quaternary groups						Interactions					
	Pure	**binary**	**100[a]**		**50**		**25**		**BHTxCC**	**BHTxPG**	**BHTxTB**	**CCxPG**	**CCxTB**	**PGxTB**
body weight (g)	***	*	1.05	ns	0.95	*	0.96	ns			Q**			L***
total liver weight (g)	Ns	*	1.22	***	1.01	Ns	1.00	ns	Qo					
Relative liver weight (g/100 g body weight)	Ns	Ns	1.03	ns	1.03	Ns	1.03	ns						
CYP (nmol/mg)	ns	ns	1.08	ns	1.00	Ns	0.96	ns	Q*			Lo		
7PR (pmol/min/mg) (+ CC25 indicator variable)	***	***	11.50	***	2.72	***	0.94	ns	Q***			Q***	Q***	L***
7PR(pmol/min/mg) (- CC25)	***	***	0.96	*	0.64	**	0.66	**	Q***			L*	Lo	L***
7ER (pmol/minute/mg)	ns	ns	0.99	ns	1.04	Ns	0.82	ns			Q*			
CDNB (µmol/minute/mg)	**	ns	0.86	ns	0.95	Ns	1.17	ns	L*	L*	Q***	L*	Lo	
DCNB (nmol/minute/mg)	***	***	0.43	***	0.82	*	1.01	ns	Q*	Q**	Q*	Qo	Q***	
CYP2B1 (fold induction) (+ PG50 indicator variable)	***	o	3.53	*	1.29	Ns	1.66	ns	L**	L***			Q**	
CYP2B1 (fold induction) (- PG50)	***	ns	1.10	ns	0.99	Ns	1.62	ns	Lo			L***	Q*	L**
CYP1A2 (fold induction)	***	***	3053	***	2.94	***	0.45	**	L*	Q***	Q***	Q***	Q***	Q***

ns: P>0.10; o: P < 0.10; *: P < 0.05; **: P < 0.01; *** P < 0.001

[a]These number refer to the total dosages in the quaternary mixtures

There are two columns under the heading ‘non-additivity’. The first one just reproduces the corresponding right column from Table S-5. It assesses non-additivity of the binary and quaternary dosage groups based on an additivity surface calculated from the single compounds and the control. The second one assesses non-additivity of the binary and quaternary dosage groups based on an additivity surface calculated from the single compounds, *binary mixtures* and controls. It is important to note that these are overall tests in a two-stage procedure. We discourage looking at departures from additivity of individual treatment groups if the overall test is not significant.

The six columns on the right in Table S-6 characterise the compounds whose effects are non-additive. In each column, there can either be an L for a ‘linear’ departure from additivity, or a Q for a ‘quadratic’ departure. A linear departure implies a non-addition that is not dose-dependent, one single term in the equation suffices to model non-addition in the two binary exposures. A quadratic departure implies a dose dependence of the departure from additivity.

As regards the calculations underpinning the last six columns of Table S-6, the departure from additivity was modelled by extending the additivity surface with product terms and squared product terms.

Then, the squared product terms with P > 0.10 was dropped. Finally, the simple product terms with P > 0.10 were dropped. Results for body weight are given below (Table S-7); results for all parameters are in the Supplementary Electronic File ‘DF6_interaction.out’.

Table S-7.

```
***** Regression Analysis *****

 Response variate: BW
     Fitted terms: Constant + BHT + CC + PG + TB + BHT2 + TB2 +
                   LL['BHTTB'] + LL['PGTB'] + QQ['BHTTB'] + i['all100'] +
                   i['all50'] + i['all25']


*** Estimates of parameters ***

                               estimate         s.e.    t(159)  t pr.
Constant                         5.6929       0.0106    534.69  <.001
BHT                             -0.1622       0.0556     -2.91  0.004
CC                              -0.0095       0.0205     -0.46  0.644
PG                              -0.0223       0.0214     -1.04  0.300
TB                               0.0594       0.0638      0.93  0.353
BHT2                             0.1509       0.0627      2.41  0.017
TB2                             -0.2073       0.0694     -2.99  0.003
LL['BHTTB']                      1.686        0.648       2.60  0.010
LL['PGTB']                      -0.388        0.118      -3.28  0.001
QQ['BHTTB']                     -7.77         2.73       -2.84  0.005
i['all100']                      0.0461       0.0356      1.30  0.197
i['all50']                      -0.0495       0.0250     -1.98  0.049
i['all25']                      -0.0389       0.0244     -1.59  0.113
```

For body weight, non-additivities for binary mixtures of PG and TB, and for BHT and TB were found, respectively. To deal with the first of these non-additivities adequately, one should add the term 0.388 PG.TB to the modelling equation. Note that this single term adequately handles the non-additivities for both the PG_TB100 and PG_TB50 groups. In other words, the non-additivity is not dose dependent.

For the non-additivity of BHT and TB, two separate terms, viz., 1.686 BHT.TB – 7.77 $(BHT.TB)^2$ need to be added to the equation. So there is a (negative) dose-dependence of the non-additivities. This comes as no surprise, because the log-body weights in the BHT_TB50 group are 0.081 ± 0.0366 ($P < 0.05$) more than additive, while in the BHT_TB100 group, the body weights are compatible with effect addition (–0.011 ± 0.040 less than additive; $P > 0.10$).

The results on body weight also show how the quaternary groups deviate from a prediction based on an additivity surface augmented with the significant terms for departures from additivity. Table S-6 also includes an overall significance test for the three quaternary exposures (for body weight, $P < 0.05$). The table also includes multiplication factors for each of the quaternary groups. For example, the all 50 group has a body weight which is a factor 0.95 lower than expected on the basis of the augmented additivity surface. Note that the results in 'interaction.out' are on ln body weights – hence different figures for the quaternary groups.

For 7PR and CYP2B1, there are two modelling strategies. The first one considers CC25 (or PG50) as a single and active dose for CC (PG). The second strategies considers CC25 (or PG50) as an aberrant group and effectively disregards the group in calculating the additivity surface or augmented additivity surface. For 7PR, the non-additivity is less extreme if the CC25 group is disregarded. For CYP2B1, the results are problematic. Considering PG50 as the active dose, a BHT.PG model term is needed to handle departures from additivity in the BHT/PG binary groups. If the PG50 group is disregarded, CC.PG and PG.TB terms are needed, but no BHT.PG term. It seems hard to draw any definite conclusion on the working of PG on this particular parameter.

9. Testing for dose addition

In the previous section effect addition was studied by comparing the parameters in binary exposure groups with predictions based by adding the response as described by the dose-response curves of the single exposures. This is a sensible approach, according to classical definitions in mixture toxicology if the compounds have an independent joint action. If, however the

compounds have a similar action, it would make more sense to make predictions based on dose addition.

Consider as an example the BHT_PG100 group, with both BHT and PG at 50% of their maximum doses. Instead of comparing this group with BHT50 and PG50, to monitor for effect addition, the response could also be related to the BHT100 and PG100 group, respectively to monitor for dose addition (see also Figure 1 in the Manuscript). If the response of some parameter to the maximum BHT dose is b, and the response to the maximum PG dose is p, it can be hypothesised that 1 unit of BHT is equivalent with b/p units of PG. If this hypothesis is correct, 0.5 units each of BHT and PG would result in a response halfway between b and p. This is the prediction under dose addition.

Table S-8 gives results on departures from dose addition in binary and quaternary groups with 100% total dose. There are 6 binary exposures with this total dose. The column 'overall' gives the statistical significance of departures in these groups, with a more detailed assessment following in the six columns right after this one. The figures in these 6 columns are multiplication factors. For example, 7PR in the BHT_CC100 group is 4.58 times larger than one would expect if dose addition would apply.

For the quaternary exposures, the response may be predicted taking into account the departures from dose addition in the binary groups with a linear regression equation of the following form.

$$\begin{aligned}(\text{response}) = \quad & \beta_b BHT + \beta_c CC + \beta_p PG + \beta_t TB + \\ & \beta_{bc} BHT.CC + \beta_{bp} BHT.PG + \beta_{bt} BHT.TB + \\ & \beta_{cp} CC.PG + \beta_{ct} CC.TB + \beta_{pt} PG.TB\end{aligned}$$

Table S-8. Departures from dose-addition in binary and quaternary groups with 100% total dose

	overall	BHTxCC	BHTxPG	BHTxTB	CCxPG	CCxTB	PGxTB	Quaternary
body weight (g)	***	1.34^{***}	1.05^{ns}	1.23^{***}	1.11^{**}	1.25^{***}	1.02^{ns}	1.49^{***}
total liver weight (g)	*	1.15^{*}	1.04^{ns}	1.13^{*}	1.08^{ns}	1.18^{**}	1.04^{ns}	1.29^{***}
Relative liver weight (g/100 g body weight)	*	1.13^{**}	1.05^{ns}	1.10^{*}	1.05^{ns}	1.08^{o}	1.08^{*}	1.07^{*}
CYP (nmol/mg)	ns	0.94^{ns}	0.97^{ns}	0.99^{ns}	0.92^{o}	0.99^{ns}	1.03^{ns}	1.01^{ns}
7PR (pmol/min/mg)	***	4.58^{***}	4.50^{***}	3.72^{***}	2.20^{***}	1.46^{o}	0.84^{ns}	1.19^{ns}
7ER (pmol/minute/mg protein)	ns	1.00^{ns}	0.97^{ns}	0.95^{ns}	0.98^{ns}	1.05^{ns}	1.19^{*}	1.50^{***}
CDNB (μmol/minute/mg protein)	*	1.37^{*}	1.29^{*}	1.14^{ns}	1.25^{o}	1.25^{o}	1.01^{ns}	1.21^{o}
DCNB (nmol/minute/mg protein)	***	1.79^{***}	1.14^{ns}	1.12^{ns}	1.26^{**}	1.33^{**}	1.09^{ns}	1.37^{***}
CYP2B1 (fold induction)	***	19.49^{***}	13.23^{***}	8.52^{***}	30.11^{***}	1.86^{ns}	2.23^{o}	1.05^{ns}
CYP1A2 (fold induction)	***	2.01^{**}	0.48^{**}	0.82^{ns}	0.54^{**}	1.01^{ns}	1.02^{ns}	1.26^{ns}

ns: P>0.10; o: P < 0.10; *: P < 0.05; **: P < 0.01; *** P < 0.001

Table S-9. Departures from dose-addition in binary and quaternary groups with 50 % total dose

	overall	BHTxCC	BHTxPG	BHTxTB	CCxPG	CCxTB	PGxTB	Quaternary
body weight (g)	***	1.24^{***}	1.44^{***}	1.12^{***}	1.28^{***}	1.04^{ns}	1.06^{*}	1.09^{**}
total liver weight (g)	*	1.04^{ns}	1.17^{**}	1.09^{o}	1.13^{*}	1.07^{ns}	1.05^{ns}	1.04^{ns}
Relative liver weight (g/100 g body weight)	o	1.02^{ns}	1.09^{*}	1.04^{ns}	1.07^{*}	1.05^{ns}	1.05^{ns}	1.00^{ns}
CYP (nmol/mg)	*	0.86^{***}	0.94^{ns}	0.95^{ns}	0.95^{ns}	0.97^{ns}	0.98^{ns}	1.02^{ns}
7PR (pmol/min/mg)	***	2.35^{***}	1.85^{***}	1.18^{ns}	1.09^{ns}	0.95^{ns}	0.76^{*}	0.71^{*}
7ER (pmol/minute/mg protein)	ns	1.04^{ns}	1.21^{ns}	1.13^{ns}	1.03^{ns}	0.81^{ns}	1.01^{ns}	1.09^{ns}
CDNB (μmol/minute/mg protein)	ns	1.12^{ns}	1.07^{ns}	1.29^{*}	1.25^{o}	1.19^{ns}	1.06^{ns}	0.89^{ns}
DCNB (nmol/minute/mg protein)	***	1.51^{***}	1.53^{***}	1.16^{o}	1.39^{***}	1.40^{***}	1.07^{ns}	1.19^{*}
CYP2B1 (fold induction)	***	6.72^{***}	1.97^{ns}	1.93^{ns}	2.16^{o}	0.85^{ns}	0.41^{*}	1.18^{ns}
CYP1A2 (fold induction)	***	1.54^{ns}	0.34^{***}	0.29^{***}	0.18^{***}	0.16^{***}	0.20^{***}	1.54^{ns}

ns: P>0.10; o: P < 0.10; *: P < 0.05; **: P < 0.01; *** P < 0.001

In this formula, the compound's concentrations are between 0 and 1. In single exposures, one of the concentrations is 1 and the remaining ones are 0. In the binary exposures, there are two concentrations with a value of 0.5 each; the other two are 0. In the groups under consideration, the concentrations sum to 1. The prediction can be compared with the realization. In the column headed 'complex', the discrepancies are listed, with their statistical significance. Table S-9 gives the results from the groups with 50% of total dose. Here, the concentrations were recorded such that the mid-value single exposures have concentration 1.

Some general conclusions are from this modeling summarized below. Departures from dose addition were observed with factors larger than 2 or smaller than 0.5 for 7PR, CYP2B1, and CYP1A2. For CYP1A2, the prediction based on the above equation with product terms agrees well with the realization in the quaternary group. This is an improvement on the corresponding result in Table S-6, in which a multiplication factor of 3053 was observed when the actual data were compared to the response additivity surface. In other words, when CYP1A2 mRNA expression was considered for the quaternary mixture, a model describing dose addition is more in line than a model describing effect addition. For the other parameters the results in the quaternary groups are neither better nor worse explained by the augmented dose additivity surface as by the augmented effect additivity surface. For 7PR, the departures from effect addition are more extreme than those for dose addition. For CYP2B it is the the other way round.

Table S-10. Body weight, liver weight, relative liver weight assessed in Sprague Dawley rats exposed for 28 days to single compounds and binary and quaternary mixtures of BHT, CC, PG and TB.

Group	Body weight (BW) (g)			Total liver weight (LW) (g)			relative liver weight (RLW) (g/100 g BW)		
	average measured ± SD[1]	geometrical mean ('observed')[2]	predicted[3]	average measured ± SD	geometrical mean ('observed')	predicted	average measured ± SD	geometrical mean ('observed')	predicted
1: Control	299,00 ± 16,76	298,56	298,57	11,13 ± 1,16	11,08	11,12	3,72 ± 0,23	3,71	3,79
2: BHT25	289,50 ± 17,43	289,06	288,09	11,94 ± 1,02	11,90	11,76	4,12 ± 0,26	4,12	4,02
3: BHT50	282,67 ± 15,60	282,31	283,01	12,16 ± 1,00	12,13	12,50	4,31 ± 0,31	4,30	4,30
4: BHT100	293,33 ± 15,44	293,00	292,92	14,43 ± 1,36	14,38	14,21	4,91 ± 0,24	4,91	4,93
5: CC25	303,83 ± 12,46	303,62	297,46	11,42 ± 1,46	11,34	11,15	3,75 ± 0,38	3,74	3,79
6: CC50	294,33 ± 16,21	293,96	296,29	11,34 ± 1,20	11,29	11,19	3,85 ± 0,23	3,84	3,80
7: CC100	294,67 ± 27,02	293,61	293,95	11,25 ± 1,57	11,16	11,26	3,81 ± 0,25	3,80	3,81
8: PG25	291,50 ± 6,75	291,43	295,14	10,81 ± 0,67	10,80	10,99	3,71 ± 0,23	3,70	3,75
9: PG50	294,33 ± 14,15	294,04	291,55	10,96 ± 1,11	10,91	10,85	3,72 ± 0,26	3,71	3,71
10: PG100	285,67 ± 18,89	285,15	285,55	10,08 ± 1,62	10,64	10,62	3,52 ± 0,43	3,67	3,65
11: TB25	291,00 ± 11,12	290,82	296,77	11,77 ± 1,01	11,74	11,49	4,05 ± 0,37	4,04	3,81
12: TB50	293,67 ± 20,89	293,02	288,64	11,27 ± 1,47	11,19	11,37	3,83 ± 0,32	3,82	3,83
13: TB100	256,17 ± 21,87	255,39	256,03	9,82 ± 0,98	9,78	9,76	3,83 ± 0,18	3,83	3,88
14: BHT_CC100	282,33 ± 23,21	281,55	281,05	12,61 ± 1,89	12,49	12,50	4,45 ± 0,36	4,44	4,28
15: BHT_PG100	287,50 ± 11,64	287,31	276,58	12,54 ± 1,02	12,50	12,19	4,36 ± 0,28	4,35	4,21
16: BHT_TB100	271,00 ± 14,64	270,67	273,63	12,13 ± 1,46	12,06	12,73	4,48 ± 0,50	4,46	4,33
17: CC_PG100	295,17 ± 13,23	294,92	289,78	11,32 ± 0,82	11,29	10,93	3,84 ± 0,29	3,83	3,73
18: CC_TB100	291,17 ± 15,28	290,84	286,73	11,47 ± 0,43	11,47	11,44	3,95 ± 0,19	3,94	3,84
19: PG_TB100	261,50 ± 19,03	260,91	282,61	10,46 ± 1,22	10,40	11,12	4,00 ± 0,38	3,98	3,76
20: all_100	302,83 ± 29,22	314,32	283,04	12,79 ± 1,83	13,48	12,00	4,21 ± 0,26	4,20	4,00
21: BHT_CC50	279,83 ± 14,46	279,53	286,94	10,97 ± 0,86	10,95	11,80	3,92 ± 0,18	3,92	4,03
22: BHT_PG50	290,00 ± 16,92	289,59	284,70	11,62 ± 1,25	11,57	11,68	4,00 ± 0,27	3,99	4,00
23: BHT_TB50	300,17 ± 27,23	310,22	286,09	12,63 ± 1,67	12,54	12,17	4,20 ± 0,28	4,19	4,05
24: CC_PG50	294,50 ± 14,35	294,20	294,21	11,33 ± 1,18	11,28	11,03	3,84 ± 0,28	3,83	3,76
25: CC_TB50	296,33 ± 10,29	296,19	295,63	11,93 ± 0,89	11,90	11,52	4,02 ± 0,21	4,02	3,82
26: PG_TB50	290,33 ± 17,64	289,90	293,31	11,38 ± 1,44	11,31	11,35	3,91 ± 0,32	3,90	3,77
27: all_50	283,17 ± 20,74	282,53	290,33	11,48 ± 1,18	11,42	11,64	4,05 ± 0,32	4,04	3,90
28: all_25	285,17 ± 23,68	284,36	294,03	11,42 ± 1,14	11,37	11,42	4,00 ± 0,20	4,00	3,85

[1]average measured is based upon the actual measured values, excluding outlier animals indiciated in Table S-1

[2]geometrical mean is e raised to the power of the average of all natural logarithms of the individual animal values

[3]predicted vales are based upon the additivity surface equations:

$\ln(BW)=5.699-0.197[BHT]-0.0156[CC]-0.0446[PG]+0.0181[TB]+ 0.1779[BHT]^2-0.1718[TB]^2$.

$\ln(LW)= 2.4088+0.2449[BHT]+0.0122[CC] -0.0463[PG]+ 0.218[TB]-0.349[TB]^2$.

$\ln(RLW)= 1.3322 +0.2634[BHT]+0.005[CC]-0.0378[PG]+0.023[TB]$.

Table S-11. Total CYP content assessed in Sprague Dawley rats exposed for 28 days to single compounds and binary and quaternary mixtures of BHT, CC, PG and TB.

Group	CYP450 (nmol/mg)			CYP450 (nmol/g liver)			CYP450 (nmol/LW/kg BW)		
	average measured ± SD[1]	geometrical mean ('observed')[2]	predicted[3]	average measured ± SD	geometrical mean ('observed')	predicted	average measured ± SD	geometrical mean ('observed')	predicted
1: Control	0,73 ± 0,07	0,73	0,73	26,66 ± 3,27	26,47	26,78	989,01 ± 123,65	981,95	1011,92
2: BHT25	0,76 ± 0,05	0,75	0,78	29,32 ± 2,66	29,22	30,79	1204,06 ± 56,99	1202,93	1236,96
3: BHT50	0,85 ± 0,08	0,85	0,84	38,21 ± 6,07	37,80	35,88	1645,73 ± 281,13	1624,32	1540,59
4: BHT100	0,99 ± 0,12	0,98	0,98	48,94 ± 5,09	48,71	49,34	2402,05 ± 266,46	2389,75	2435,48
5: CC25	0,75 ± 0,05	0,75	0,75	28,03 ± 4,60	27,72	27,34	1048,96 ± 186,03	1035,59	1035,22
6: CC50	0,80 ± 0,06	0,80	0,76	29,91 ± 3,47	29,74	27,94	1150,61 ± 147,28	1142,35	1060,29
7: CC100	0,77 ± 0,07	0,77	0,79	28,44 ± 3,98	28,22	29,20	1082,99 ± 162,34	1072,94	1112,98
8: PG25	0,75 ± 0,12	0,75	0,72	27,12 ± 4,57	26,78	27,47	1007,04 ± 185,50	991,80	1019,51
9: PG50	0,78 ± 0,10	0,77	0,71	30,49 ± 5,04	30,15	28,22	1132,78 ± 195,80	1118,74	1027,64
10: PG100	0,77 ± 0,08	0,76	0,69	28,75 ± 1,78	28,70	29,54	1013,37 ± 152,55	1002,57	1041,59
11: TB25	0,77 ± 0,04	0,77	0,80	30,35 ± 3,09	30,21	31,69	1222,73 ± 94,71	1219,50	1230,95
12: TB50	0,81 ± 0,04	0,81	0,87	36,29 ± 2,76	36,20	34,96	1389,88 ± 161,70	1382,27	1372,94
13: TB100	0,75 ± 0,07	0,75	1,02	34,20 ± 4,35	33,95	34,18	1307,16 ± 141,07	1300,52	1301,93
14: BHT_CC100	0,82 ± 0,05	0,82	0,87	38,17 ± 4,18	37,99	36,88	1698,17 ± 226,90	1685,07	1579,73
15: BHT_PG100	0,84 ± 0,06	0,84	0,82	37,87 ± 0,96	37,86	37,59	1650,14 ± 98,99	1647,70	1554,32
16: BHT_TB100	0,79 ± 0,17	0,85	0,99	37,86 ± 8,50	40,26	46,42	1663,25 ± 240,71	1648,41	2063,10
17: CC_PG100	0,71 ± 0,06	0,71	0,74	26,62 ± 2,40	26,53	29,33	1018,59 ± 78,91	1015,99	1075,08
18: CC_TB100	0,76 ± 0,07	0,76	0,90	34,37 ± 3,21	34,23	36,39	1352,15 ± 88,83	1349,71	1434,98
19: PG_TB100	0,78 ± 0,06	0,78	0,84	30,85 ± 1,95	30,79	36,65	1231,32 ± 116,26	1226,91	1390,16
20: all_100	0,79 ± 0,05	0,79	0,85	34,02 ± 4,78	33,74	37,52	1436,91 ± 262,52	1416,95	1520,23
21: BHT_CC50	0,72 ± 0,06	0,72	0,80	28,97 ± 5,13	28,60	31,48	1135,80 ± 213,54	1119,94	1268,00
22: BHT_PG50	0,78 ± 0,07	0,78	0,78	32,44 ± 6,26	31,95	31,77	1289,91 ± 216,92	1275,93	1260,91
23: BHT_TB50	0,79 ± 0,08	0,78	0,85	33,15 ± 5,16	32,81	36,63	1384,35 ± 167,59	1375,84	1515,59
24: CC_PG50	0,76 ± 0,04	0,76	0,74	30,26 ± 2,36	30,19	28,01	1166,74 ± 170,45	1156,92	1043,06
25: CC_TB50	0,78 ± 0,05	0,78	0,81	32,82 ± 2,18	32,77	32,37	1317,82 ± 57,27	1316,78	1260,28
26: PG_TB50	0,78 ± 0,05	0,77	0,78	31,13 ± 3,39	30,98	32,53	1212,27 ± 104,75	1208,60	1241,11
27: all_50	0,76 ± 0,07	0,76	0,79	30,44 ± 3,50	30,27	32,26	1236,56 ± 194,52	1224,17	1267,86
28: all_25	0,73 ± 0,07	0,73	0,76	26,56 ± 3,05	26,41	29,57	1061,11 ± 114,72	1055,72	1142,70

[1]average measured is based upon the actual measured values, excluding outlier animals indiciated in Table S-1

[2]geometrical mean is e raised to the power of the average of all natural logarithms of the individual animal values

[3]predicted vales are based upon the additivity surface equations:

ln(CYP, nmol/mg)=-0.3122+0.2931[BHT]+0.0773[CC]+0.0572[PG]+ 0.335[TB]

ln (CYP, nmol/g liver)= 3.2876+0.6112[BHT]+0.0865[CC]+0.0981[PG]+0.823[TB]-0,579[TB]^2

ln(CYP, nmol/LW/kg BW)= 6.9196+0.8783[BHT]+0.0952[CC] +0.0289[PG]+ 0.969[TB]-0.717[TB]^2

Table S-12. 7ER activities assessed in Sprague Dawley rats exposed for 28 days to single compounds and binary and quaternary mixtures of BHT, CC, PG and TB.

Group	7ER (pmol/min/mg)			7ER (nmol/min/g liver)			7ER (nmol/min/rlw)		
	average measured ± SD[1]	geometrical mean ('observed')[2]	predicted[3]	average measured ± SD	geometrical mean ('observed')	predicted	average measured	geometrical mean ('observed')	predicted
1: Control	17,33 ± 3,55	16,98	17,48	0,64 ± 0,15	0,62	0,63	23,58 ± 5,57	22,96	23,58
2: BHT25	22,41 ± 6,72	21,69	19,52	0,87 ± 0,25	0,84	0,76	35,45 ± 9,27	34,60	30,32
3: BHT50	22,49 ± 6,64	21,57	22,02	0,99 ± 0,25	0,96	0,93	42,99 ± 13,29	41,16	39,91
4: BHT100	28,38 ± 5,56	27,90	28,30	1,40 ± 0,27	1,38	1,43	69,04 ± 14,33	67,74	70,83
5: CC25	15,50 ± 3,77	15,09	17,32	0,58 ± 0,15	0,56	0,63	21,62 ± 5,92	20,82	23,65
6: CC50	19,82 ± 2,08	19,73	17,16	0,74 ± 0,08	0,73	0,63	28,47 ± 4,18	28,22	23,73
7: CC100	16,36 ± 2,16	16,24	16,83	0,60 ± 0,09	0,59	0,63	22,96 ± 4,49	22,61	23,89
8: PG25	19,03 ± 5,20	18,44	18,23	0,69 ± 0,23	0,66	0,68	25,72 ± 9,41	24,51	25,36
9: PG50	17,39 ± 2,48	17,22	17,37	0,68 ± 0,08	0,67	0,66	25,28 ± 4,13	24,94	24,29
10: PG100	12,97 ± 1,53	12,90	12,88	0,49 ± 0,04	0,48	0,49	17,14 ± 2,95	16,92	17,00
11: TB25	21,69 ± 2,91	21,51	23,25	0,86 ± 0,16	0,85	0,99	34,32 ± 4,11	34,12	38,27
12: TB50	36,24 ± 2,45	36,17	31,07	1,62 ± 0,18	1,62	1,44	62,29 ± 8,96	61,68	56,67
13: TB100	52,94 ± 9,58	52,25	55,30	2,38 ± 0,33	2,36	2,41	91,39 ± 15,08	90,39	91,68
14: BHT_CC100	18,02 ± 2,90	17,84	21,35	0,83 ± 0,15	0,82	0,91	37,03 ± 7,03	36,50	39,08
15: BHT_PG100	17,82 ± 3,04	17,60	21,91	0,80 ± 0,09	0,80	0,98	34,85 ± 4,02	34,66	41,02
16: BHT_TB100	32,72 ± 6,79	32,21	38,93	1,56 ± 0,26	1,54	2,12	69,54 ± 13,32	68,62	94,60
17: CC_PG100	13,66 ± 1,57	13,58	17,24	0,51 ± 0,09	0,51	0,67	19,65 ± 3,37	19,38	24,73
18: CC_TB100	28,09 ± 1,67	28,05	30,32	1,27 ± 0,08	1,26	1,43	49,94 ± 3,16	49,86	56,61
19: PG_TB100	29,91 ± 2,87	29,79	30,96	1,18 ± 0,11	1,17	1,52	47,23 ± 7,42	46,76	58,50
20: all_100	28,88 ± 8,42	27,88	26,41	1,25 ± 0,41	1,19	1,25	53,05 ± 19,77	49,94	51,42
21: BHT_CC50	20,36 ± 8,72	18,79	19,37	0,82 ± 0,41	0,75	0,76	32,45 ± 16,93	29,37	30,51
22: BHT_PG50	22,03 ± 12,36	19,53	20,48	0,95 ± 0,63	0,80	0,83	37,55 ± 23,91	32,13	33,04
23: BHT_TB50	32,66 ± 12,20	30,91	26,11	1,37 ± 0,53	1,30	1,20	57,66 ± 22,47	54,31	49,76
24: CC_PG50	18,38 ± 9,20	16,68	18,06	0,74 ± 0,37	0,67	0,68	28,79 ± 15,65	25,50	25,41
25: CC_TB50	20,98 ± 3,30	20,78	23,05	0,88 ± 0,15	0,88	0,99	35,48 ± 5,35	35,17	38,42
26: PG_TB50	24,52 ± 2,72	24,39	24,27	0,98 ± 0,14	0,98	1,07	38,31 ± 4,36	38,11	41,24
27: all_50	23,35 ± 3,87	23,09	21,89	0,90 ± 0,21	0,88	0,92	36,20 ± 7,72	35,44	36,32
28: all_25	16,49 ± 2,91	16,28	19,65	0,60 ± 0,11	0,59	0,77	23,94 ± 4,23	23,62	29,61

[1]average measured is based upon the actual measured values, excluding outlier animals indiciated in Table S-1

[2]geometrical mean is e raised to the power of the average of all natural logarithms of the individual animal values

[3]predicted vales are based upon the additivity surface equations:

$\ln(7ER, pmol/min/mg)=2.8613+0.4816[BHT]-0.0384[CC]+0.325[PG]+1.1514[TB]-0.631[PG]^2$.

$\ln(7ER, nmol/min/g\ liver=-0.4685+0.829[BHT]+0.502[PG]+1.995[TB]-0.755[PG]^2 -0.648[TB]^2$.

$\ln(7ER, nmol/min/rlw)=3.1603+1.1[BHT]+0.013[CC]+0.494[PG]+2.153[TB]-0.821[PG]^2-0.795[TB]^2$.

Table S-13. 7PR activities assessed in Sprague Dawley rats exposed for 28 days to single compounds and binary and quaternary mixtures of BHT, CC, PG and TB.

Group	7PR (pmol/min/mg)			7PR (nmol/min/g liver)			7PR (nmol/min/rlw)		
	average measured ± SD[1]	geometrical mean ('observed')[2]	predicted[3#]	average measured ± SD	geometrical mean ('observed')	predicted	average measured ± SD	geometrical mean ('observed')	predicted
1: Control	9,50 ± 0,87	9,46	10,07	0,35 ± 0,03	0,34	0,37	12,90 ± 1,72	12,79	14,10
2: BHT25	68,07 ± 11,81	67,24	60,55	2,66 ± 0,63	2,60	2,42	109,22 ± 23,44	107,24	98,37
3: BHT50	235,55 ± 64,36	227,49	245,15	10,56 ± 3,22	10,10	10,64	459,00 ± 161,72	434,02	461,66
4: BHT100	702,50 ± 126,50	693,07	685,40	34,79 ± 6,63	34,30	34,02	1699,42 ± 267,47	1682,81	1667,53
5: CC25	54,50 ± 33,87	44,67	44,66	2,10 ± 1,53	1,65	1,65	78,82 ± 57,77	61,63	61,63
6: CC50	11,06 ± 1,87	10,93	10,07	0,41 ± 0,08	0,41	0,37	15,98 ± 3,50	15,63	14,10
7: CC100	9,67 ± 1,20	9,61	10,07	0,35 ± 0,05	0,35	0,37	13,57 ± 2,45	13,38	14,10
8: PG25	14,13 ± 1,52	14,07	13,99	0,51 ± 0,09	0,50	0,52	18,89 ± 2,91	18,70	19,64
9: PG50	16,95 ± 5,74	16,18	16,25	0,66 ± 0,23	0,63	0,61	24,57 ± 8,44	23,44	22,57
10: PG100	13,58 ± 4,13	13,07	13,07	0,52 ± 0,18	0,49	0,49	18,21 ± 7,13	17,15	17,29
11: TB25	11,89 ± 3,81	11,40	11,57	0,47 ± 0,19	0,45	0,45	19,09 ± 6,97	18,08	17,28
12: TB50	15,93 ± 6,11	14,99	13,33	0,72 ± 0,29	0,67	0,55	27,56 ± 11,03	25,57	21,25
13: TB100	17,41 ± 5,83	16,69	17,64	0,79 ± 0,28	0,75	0,83	30,18 ± 10,34	28,88	32,04
14: BHT_CC100	240,81 ± 66,45	232,24	218,93	11,28 ± 3,83	10,71	9,43	502,69 ± 182,18	475,14	406,53
15: BHT_PG100	378,89 ± 119,02	362,52	382,23	17,13 ± 5,51	16,40	16,99	747,05 ± 241,58	713,72	712,25
16: BHT_TB100	325,22 ± 159,88	286,56	302,81	15,45 ± 7,51	13,70	14,77	662,51 ± 264,42	610,50	644,37
17: CC_PG100	25,96 ± 13,07	23,25	16,14	0,97 ± 0,48	0,87	0,61	37,40 ± 19,16	33,18	22,46
18: CC_TB100	19,08 ± 10,23	16,94	13,29	0,88 ± 0,52	0,76	0,55	34,55 ± 20,39	30,11	21,15
19: PG_TB100	12,40 ± 3,09	12,08	21,24	0,49 ± 0,10	0,48	0,90	19,26 ± 3,68	18,96	33,61
20: all_100	86,61 ± 20,11	84,82	372,09	3,76 ± 1,18	3,62	16,00	159,17 ± 54,97	151,93	630,24
21: BHT_CC50	104,99 ± 33,33	99,28	273,95	4,22 ± 1,53	3,96	11,02	165,91 ± 58,99	155,17	439,72
22: BHT_PG50	97,33 ± 21,22	95,45	89,98	3,99 ± 0,75	3,93	3,67	158,89 ± 26,87	157,07	147,94
23: BHT_TB50	67,50 ± 13,10	66,51	72,85	2,85 ± 0,70	2,79	3,10	118,95 ± 25,27	116,86	126,85
24: CC_PG50	13,20 ± 3,39	12,83	61,19	0,52 ± 0,11	0,51	2,30	19,90 ± 3,72	19,61	84,69
25: CC_TB50	12,44 ± 4,20	11,89	51,31	0,53 ± 0,18	0,50	2,01	21,01 ± 6,90	20,13	75,55
26: PG_TB50	11,21 ± 1,75	11,09	16,12	0,45 ± 0,05	0,44	0,64	17,51 ± 2,60	17,33	24,14
27: all_50	25,40 ± 8,33	24,50	33,72	1,01 ± 0,30	0,98	1,35	41,05 ± 12,82	39,63	52,77
28: all_25	13,75 ± 2,36	13,57	20,18	0,50 ± 0,10	0,49	0,78	20,05 ± 4,03	19,70	30,06

[1]average measured is based upon the actual measured values, excluding outlier animals indiciated in Table S-1

[2]geometrical mean is e raised to the power of the average of all natural logarithms of the individual animal values

[3]predicted vales are based upon the additivity surface equations:

$\ln(7PR, pmol/min/mg)=2.31+8.918[BHT]+1.623[PG]+0.56[TB]-4.698[BHT]^2-1.363[PG]^2$, with indicator variable of 1.489 for CC25, if present.

$\ln(7PR, nmol/min/g\ liver)=-0.9921+9.303[BHT]+1.699[PG]+0.803[TB]-4.784[BHT]^2-1.413[PG]^2$, with indicator variable of 1.493 for CC25, if present.

$\ln(7PR, nmol/min/rlw)=2.6461+9.6[BHT]+1.657[PG]+0.821[TB]-4.827[BHT]^2-1.453[PG]^2$, with indicator variable of 1.475 for CC25, if present.

Table S-14. CYP1A2 and CYP2B1 mRNA induction assessed in Sprague Dawley rats exposed for 28 days to single compounds and binary and quaternary mixtures of BHT, CC, PG and TB.

Group	CYP1A2 (fold induction)			CYP2B1 (fold induction)		
	average measured ± SD[1]	geometrical mean ('observed')[2]	predicted[3]	average measured ± SD	geometrical mean ('observed')	predicted
1: Control	1,00 ± 0,14	0,99	1,04	1,00 ± 0,13	0,99	0,81
2: BHT25	1,52 ± 0,64	1,42	1,12	57,88 ± 32,57	51,99	34,49
3: BHT50	1,31 ± 1,00	0,98	1,21	555,47 ± 377,80	381,87	512,17
4: BHT100	1,68 ± 0,95	1,49	1,42	1827,03 ± 1839,51	1341,33	1289,49
5: CC25	1,46 ± 1,44	0,84	1,19	10,67 ± 10,79	6,49	6,49
6: CC50	2,03 ± 1,55	1,46	1,37	3,82 ± 3,97	2,20	2,20
7: CC100	2,36 ± 1,41	1,90	1,81	1,50 ± 1,27	1,03	1,03
8: PG25	3,54 ± 0,65	3,50	2,52	0,66 ± 0,22	0,63	0,81
9: PG50	3,52 ± 0,90	3,40	4,18	3,06 ± 2,59	2,28	2,28
10: PG100	3,91 ± 1,18	3,78	3,60	0,71 ± 0,25	0,66	0,81
11: TB25	8,76 ± 2,65	8,44	6,05	1,08 ± 1,01	0,75	0,87
12: TB50	14,44 ± 6,64	13,19	16,96	1,82 ± 1,13	1,49	1,33
13: TB100	15,13 ± 5,76	14,22	13,63	9,79 ± 5,51	8,55	8,69
14: BHT_CC100	3,39 ± 1,54	3,08	1,58	396,86 ± 115,49	381,43	1107,63
15: BHT_PG100	1,19 ± 0,25	1,17	4,77	323,76 ± 134,28	302,92	1362,44
16: BHT_TB100	3,86 ± 0,83	3,78	19,70	934,18 ± 842,90	601,30	744,45
17: CC_PG100	1,67 ± 0,94	1,45	5,26	46,74 ± 64,28	22,49	6,49
18: CC_TB100	5,27 ± 1,35	5,12	21,76	11,89 ± 15,17	5,29	3,87
19: PG_TB100	8,14 ± 4,34	7,34	64,04	9,37 ± 9,26	5,15	3,68
20: all_100	3,53 ± 0,83	3,44	15,91	109,63 ± 61,83	97,98	232,69
21: BHT_CC50	2,07 ± 1,36	1,82	1,27	194,54 ± 99,02	168,44	285,63
22: BHT_PG50	0,74 ± 0,60	0,58	2,50	84,93 ± 76,96	57,06	41,86
23: BHT_TB50	0,99 ± 0,30	0,94	6,59	56,03 ± 29,37	45,76	40,71
24: CC_PG50	0,45 ± 0,29	0,35	2,77	6,11 ± 4,20	4,83	6,50
25: CC_TB50	0,70 ± 0,41	0,59	6,94	2,32 ± 2,19	1,51	6,99
26: PG_TB50	1,15 ± 0,54	1,05	14,78	0,96 ± 0,97	0,67	0,87
27: all_50	0,68 ± 0,17	0,66	4,89	32,33 ± 49,33	16,70	30,08
28: all_25	0,62 ± 0,49	0,47	2,29	14,07 ± 15,61	8,11	6,62

[1]average measured is based upon the actual measured values, excluding outlier animals indiciated in Table S-1

[2]geometrical mean is e raised to the power of the average of all natural logarithms of the individual animal values

[3]predicted vales are based upon the additivity surface equations:

$\ln(CYP1A2)=0.042+0.312[BHT]+0.551[CC]+4.16[PG]+8.59[TB]-2.92[PG]^2-6.02[TB]^2$

$\ln(CYP2B1)=-0.208+19.07[BHT]-11.7[BHT]^2-0.39[TB]+2.76[TB]^2+18.56[CC]-48.5[CC]^2+30.18[CC]^3$, with indicator variable of 1.034 present in equation if PG is present at fractional dose level of 50, in mixture

Table S-15. GST activities towards CDNB assessed in Sprague Dawley rats exposed for 28 days to single compounds and binary and quaternary mixtures of BHT, CC, PG and TB.

Group	CDNB (µmol/min/mg)			CDNB (µmol/min/g liver)			CDNB (µmol/min/rlw)		
	average measured ± SD[1]	geometrical mean ('observed')[2]	predicted[3]	average measured ± SD	geometrical mean ('observed')	predicted	average measured ± SD	geometrical mean ('observed')	predicted
1: Control	2,14 ± 0,49	2,08	2,25	221,34 ± 52,10	215,36	230,67	8215,47 ± 1933,16	7988,82	8261,82
2: BHT25	2,80 ± 0,76	2,72	2,82	279,10 ± 80,97	268,86	284,35	11399,57 ± 2967,40	11068,96	12235,73
3: BHT50	4,23 ± 0,62	4,19	3,61	437,96 ± 82,04	430,96	357,41	18742,00 ± 3101,89	18517,24	17246,95
4: BHT100	5,70 ± 0,83	5,66	6,03	539,86 ± 93,58	533,35	575,94	26539,33 ± 5196,39	26168,22	26433,83
5: CC25	2,24 ± 0,41	2,21	2,21	226,84 ± 44,69	223,00	229,63	8427,66 ± 1403,21	8329,89	8383,65
6: CC50	2,18 ± 0,49	2,12	2,17	234,71 ± 33,79	232,70	228,53	8991,14 ± 1061,97	8938,71	8513,58
7: CC100	2,14 ± 0,49	2,10	2,08	230,24 ± 48,13	225,93	226,33	8719,40 ± 1669,03	8589,18	8783,23
8: PG25	2,38 ± 0,55	2,33	2,30	248,27 ± 64,93	240,62	233,00	9101,91 ± 1981,91	8912,98	8349,29
9: PG50	2,33 ± 0,75	2,24	2,34	235,92 ± 67,03	227,98	235,50	8730,06 ± 2466,77	8459,15	8443,25
10: PG100	2,48 ± 0,34	2,46	2,42	244,92 ± 42,20	241,97	239,80	8579,93 ± 1668,10	8451,77	8605,01
11: TB25	3,27 ± 0,91	3,18	2,70	315,59 ± 91,85	305,44	276,34	12601,34 ± 3089,25	12328,19	11151,36
12: TB50	3,36 ± 0,73	3,30	3,25	346,36 ± 71,83	340,10	332,11	13167,67 ± 2518,15	12985,59	14002,54
13: TB100	4,53 ± 0,91	4,45	4,68	466,51 ± 77,80	461,30	478,52	17937,42 ± 3457,58	17668,40	17455,32
14: BHT_CC100	4,47 ± 1,64	4,20	3,38	422,75 ± 145,93	397,60	346,09	18823,59 ± 6831,80	17636,95	17246,80
15: BHT_PG100	4,74 ± 1,11	4,63	3,72	446,16 ± 102,23	436,11	362,36	19544,61 ± 5279,04	18981,27	17468,37
16: BHT_TB100	5,43 ± 1,32	5,31	5,13	517,46 ± 90,55	511,48	508,13	23085,53 ± 4165,13	22794,63	28736,48
17: CC_PG100	2,81 ± 0,38	2,78	2,24	271,65 ± 11,23	271,45	233,05	10451,34 ± 1215,99	10393,68	8685,48
18: CC_TB100	3,73 ± 0,56	3,69	3,11	386,24 ± 88,99	375,20	327,80	15123,63 ± 3124,77	14792,29	14360,23
19: PG_TB100	3,37 ± 0,70	3,31	3,34	329,09 ± 59,88	324,92	336,73	13140,34 ± 2448,70	12945,86	14208,26
20: all_100	4,76 ± 1,14	4,64	3,30	459,96 ± 95,01	451,29	335,53	19501,21 ± 4937,17	18949,91	16366,98
21: BHT_CC50	3,26 ± 0,68	3,20	2,78	316,07 ± 79,20	305,91	283,89	12336,34 ± 3018,54	11978,09	12469,54
22: BHT_PG50	3,26 ± 0,96	3,14	2,91	307,53 ± 88,73	297,45	290,67	12250,82 ± 3361,51	11880,63	12625,71
23: BHT_TB50	4,90 ± 1,07	4,81	3,41	475,05 ± 133,07	458,26	343,28	20002,94 ± 5995,28	19215,47	16725,43
24: CC_PG50	2,71 ± 0,68	2,65	2,25	262,98 ± 70,97	256,07	231,79	10029,80 ± 2472,88	9814,56	8470,01
25: CC_TB50	3,30 ± 0,90	3,19	2,65	333,14 ± 101,54	315,88	275,17	13415,93 ± 4359,61	12694,58	11325,53
26: PG_TB50	2,96 ± 0,74	2,89	2,75	281,95 ± 76,22	272,40	279,38	10960,41 ± 2854,05	10628,39	11283,54
27: all_50	3,13 ± 0,48	3,10	2,76	298,59 ± 55,91	294,21	281,30	12125,40 ± 2633,67	11897,43	12072,98
28: all_25	3,14 ± 0,25	3,13	2,51	308,54 ± 44,62	305,93	256,52	12303,43 ± 1465,69	12230,47	10172,86

[1]average measured is based upon the actual measured values, excluding outlier animals indiciated in Table S-1

[2]geometrical mean is e raised to the power of the average of all natural logarithms of the individual animal values

[3]predicted vales are based upon the additivity surface equations:

ln(CDNB, µmol/min/mg)=0.8128+0.9836[BHT]-0.0813[CC]+0.0699[PG]+0.7298[TB].

ln(CDNB, µmol/min/g liver)=5.441+0.915[BHT]-0.019[CC]+0.0388[PG]+0.7297[TB].

ln(CDNB, µmol/min/rlw)=9.0194+1.882[BHT]-0.0612[CC]+0.0407[PG]+1.364[TB]-0.719 $[BHT]^2$-0.616$[TB]^2$.

Table S-16. GST activities towards DCNB assessed in Sprague Dawley rats exposed for 28 days to single compounds and binary and quaternary mixtures of BHT, CC, PG and TB.

Group	DCNB (nmol/min/mg)			DCNB (µmol/min/g liver)			DCNB (µmol/min/rlw)		
	average measured ± SD[1]	geometrical mean ('observed')[2]	predicted[3]	average measured ± SD	geometrical mean ('observed')	predicted	average measured ± SD	geometrical mean ('observed')	predicted
1: Control	87,05 ± 9,40	86,60	90,63	9,05 ± 1,14	8,98	9,50	334,83 ± 36,33	333,10	344,12
2: BHT25	113,74 ± 10,22	113,38	109,71	11,30 ± 1,61	11,20	11,23	464,47 ± 62,81	461,10	471,24
3: BHT50	145,14 ± 20,40	144,00	135,18	15,07 ± 3,14	14,81	13,50	650,68 ± 159,36	636,32	626,66
4: BHT100	203,94 ± 37,47	201,24	208,99	19,15 ± 2,98	18,97	19,81	939,38 ± 142,84	930,63	932,62
5: CC25	92,90 ± 9,25	92,52	88,33	9,43 ± 1,43	9,34	9,32	351,73 ± 50,57	348,91	343,14
6: CC50	87,63 ± 17,51	86,34	85,98	9,57 ± 1,66	9,45	9,15	367,04 ± 59,09	363,19	342,10
7: CC100	82,68 ± 21,14	80,35	81,41	8,78 ± 1,64	8,65	8,80	335,09 ± 69,28	328,90	340,02
8: PG25	90,86 ± 16,28	89,63	94,27	9,32 ± 1,22	9,25	9,72	344,26 ± 36,19	342,67	350,95
9: PG50	105,51 ± 14,68	104,65	98,29	10,72 ± 1,43	10,64	9,96	397,88 ± 55,82	394,73	358,35
10: PG100	103,90 ± 11,70	103,38	105,51	10,26 ± 1,55	10,16	10,39	357,00 ± 44,65	354,77	371,26
11: TB25	116,82 ± 20,59	115,35	114,35	11,28 ± 2,41	11,07	10,95	451,19 ± 69,17	446,98	443,07
12: TB50	135,37 ± 15,53	134,56	135,47	13,98 ± 2,06	13,85	12,66	530,25 ± 41,15	528,91	532,55
13: TB100	157,12 ± 25,55	155,43	155,21	16,25 ± 2,47	16,11	16,90	622,45 ± 90,92	617,03	616,46
14: BHT_CC100	164,62 ± 24,05	163,17	125,5694	15,51 ± 1,61	15,44	12,77	690,30 ± 94,20	685,06	607,16
15: BHT_PG100	156,03 ± 21,16	154,81	145,4379	14,72 ± 2,04	14,59	14,07	641,09 ± 89,58	635,19	647,22
16: BHT_TB100	165,12 ± 30,28	162,36	199,6988	16,06 ± 3,86	15,63	17,82	706,38 ± 127,74	696,54	955,70
17: CC_PG100	107,38 ± 14,47	106,62	92,8527	10,41 ± 0,70	10,39	9,57	400,06 ± 46,61	397,97	355,39
18: CC_TB100	131,65 ± 27,04	129,68	128,3083	13,52 ± 3,61	13,18	12,17	529,90 ± 125,04	519,45	527,82
19: PG_TB100	132,80 ± 14,38	132,13	145,5455	13,06 ± 1,82	12,96	13,18	519,80 ± 63,56	516,52	550,65
20: all_100	156,52 ± 18,20	155,68	137,2691	15,42 ± 3,22	15,14	12,79	646,91 ± 131,41	635,71	599,69
21: BHT_CC50	138,18 ± 18,90	137,07	107,2809	13,35 ± 2,68	13,10	11,06	520,29 ± 91,68	512,87	471,74
22: BHT_PG50	142,46 ± 26,65	140,39	114,9618	13,34 ± 1,38	13,28	11,59	534,01 ± 66,40	530,42	488,19
23: BHT_TB50	157,98 ± 19,68	156,92	139,4404	15,24 ± 2,99	14,95	13,04	640,23 ± 135,45	627,02	613,04
24: CC_PG50	110,01 ± 22,08	108,41	91,6238	10,52 ± 0,86	10,49	9,53	403,31 ± 35,02	402,09	349,53
25: CC_TB50	154,30 ± 34,62	150,89	111,4462	15,05 ± 1,88	14,95	10,75	605,61 ± 80,29	600,68	441,99
26: PG_TB50	122,09 ± 16,62	121,05	119,0879	11,62 ± 2,30	11,42	11,22	450,38 ± 71,24	445,61	452,38
27: all_50	128,28 ± 16,79	127,33	113,7023	12,21 ± 1,84	12,10	11,12	492,03 ± 56,64	489,36	468,35
28: all_25	114,01 ± 21,53	112,36	102,3271	11,12 ± 2,06	10,97	10,33	445,87 ± 92,31	438,64	407,43

[1]average measured is based upon the actual measured values, excluding outlier animals indiciated in Table S-1

[2]geometrical mean is e raised to the power of the average of all natural logarithms of the individual animal values

[3]predicted vales are based upon the additivity surface equations:

ln(DNCB, nmol/min/mg)=4.5068+0.8355[BHT]-0.1073[CC]+0.152[PG]+1.071[TB]-0.533[TB]^2.

ln (DNCB, µmol/min/g liver)=2.2508+0.7355[BHT]-0.0764[CC]+0.0896[PG]+0.5763[TB].

ln (DNCB, µmol/min/rlw)=1.487-0.012[CC]+0.0759[PG]+1.165[TB]-0.490 [BHT]^2-0.582[TB]^2.