ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Introduction of Flavor Chemical Eugenol Attenuating the Synergistic Toxicological Interactions of Flavor Mixtures

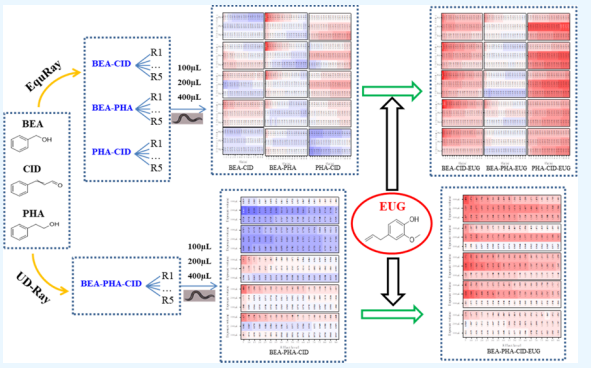
Sheng Lu, Shu-Shen Liu,* Peng Huang, and Ze-Jun Wang

Cite This: *ACS Omega* 2022, 7, 32238−32249

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: The flavor chemicals benzyl alcohol (BEA), phenylethanol (PHA), and cinnamaldehyde (CID) and their binary mixtures have high toxicity sensitivity to the lethal endpoint of *Caenorhabditis elegans*. Some binary flavor mixtures even have synergistic toxicological interactions. Eugenol (EUG) is closely related to human life and has many special nonlethal effects on organisms. The effect of its introduction on the combined toxicities of flavor mixtures is worth studying. We introduced EUG into three binary (BEA-PHA, BEA-CID, and PHA-CID) and one ternary (BEA-PHA-CID) flavor mixture systems. Five representative mixture rays were selected from each of the four mixture systems using the uniform design ray (UD-Ray) method. The lethal toxicity of each mixture ray to *C. elegans* was measured at three different exposure volumes (100, 200, and 400 μL), and a dose−effect model was established. The new parameter iSPAN was used to quantitatively characterize the toxicity sensitivity of each chemical and mixture ray. The toxicological interaction of each mixture was evaluated by the toxicological interaction heatmap based on the combination index (CI). It can be seen that all flavor chemicals and their ternary and quaternary mixture rays have high iSPANs, and the highest value is 16.160 (BEA-PHA-CID-EUG-R1 at 400 μL). According to the heatmap and CI, the introduction of EUG attenuates the synergistic toxicological interactions of flavor mixtures, leading to the transformation ofsynergistic interactions in flavor mixtures into additive action and even antagonistic interaction, and the CI value of the antagonistic interaction is up to 1.8494 (BEA-CID-EUG-R4 at 400 μL).



1. INTRODUCTION

Flavor chemicals are closely related to human life, and are often used in personal care products, which are regarded as emerging pollutants in the environment. Due to the daily behavior of human beings, such as washing and swimming, these flavor chemicals enter the environment and cause certain harm to the environment;[1] meanwhile, excessive intake of flavor chemicals will also cause harm to human health.[2] However, flavor chemicals usually appear in the form of mixtures; considering the impact on human health and the ecological environment, it is necessary to study the combined toxicities of flavor mixtures in addition to single chemicals. The analysis of previous research results shows that for any toxic chemical, its toxicity to an organism along with the change in the concentration degree is different; we call it toxicity sensitivity. For quantitative evaluation, we designed a new parameter iSPAN; the larger the value, the more significant the change in toxicity when the concentration changes slightly. Our previous studies showed that three common flavor chemicals, benzyl alcohol (BEA), phenylethanol (PHA), and cinnamaldehyde (CID), as well as the binary mixture rays composed of these three flavor compounds, had significant toxic effects on *Caenorhabditis elegans*. At the same time, the iSPANs of the three flavor chemicals and their binary mixture rays are larger than those of other substances such as pesticides, substituted phenols, and ionic liquids, and the combined toxicity and iSPAN to *C. elegans* were also affected by different exposure volumes.[1] However, further research is needed to determine whether this conclusion is still valid for multiflavor mixture systems.

Eugenol (EUG) is a common flavor chemical, which as a natural substance in cloves is also present in other types of aromatic plants, such as basil, cinnamon, and bay leaf, and is the main extract of cloves.[3] Studies have reported that EUG is often used for fish anesthesia, such as freshwater fish. Because water containing EUG is used in the process of fish anesthesia, arbitrary discharge may form wastewater that will pollute the environment;[4] therefore, special attention should be paid to the use of EUG. EUG is often used as a flavoring agent in food, in cosmetics to add fragrance, and as a component of pesticides

Received: June 8, 2022
Accepted: August 23, 2022
Published: September 1, 2022

© 2022 The Authors. Published by American Chemical Society

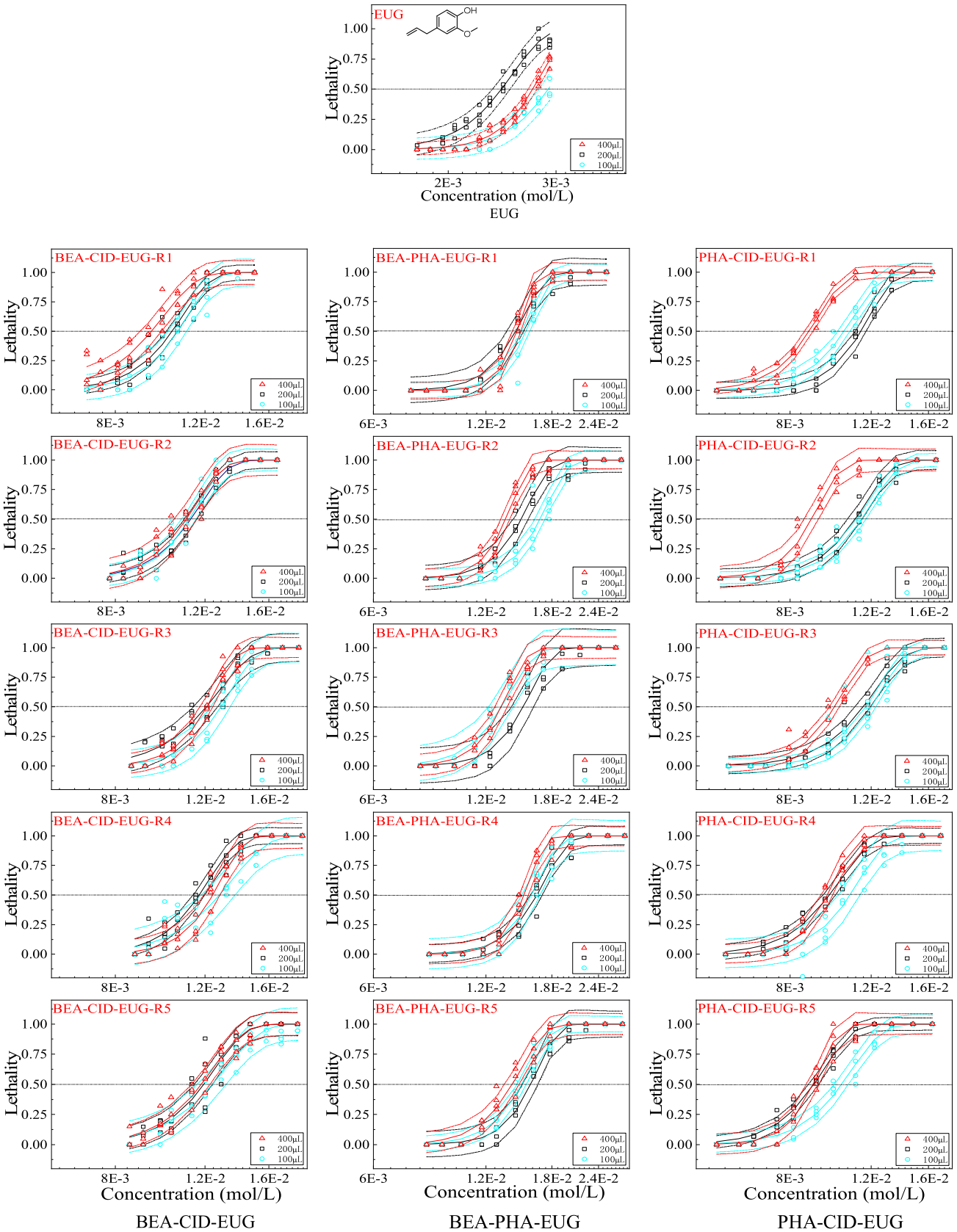


Figure 1. Concentration−response curves of EUG and 15 mixture rays in BEA-CID-EUG, BEA-PHA-EUG, and PHA-CID-EUG systems at three exposure volumes, where — refers to the fitting curves and ··· refers to the 95% observation-based confidence intervals (OCIs).

used in agriculture;[5−7] nevertheless, it is also harmful to human health and the ecological environment. EUG also appears in traditional medicine as a preservative or an antibacterial agent in many Asian countries or as a dental cavity filler[8,9] in the

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249

Table 1. Weibull Fitting Parameters (α and β), Fitting Statistics (R^2 and RMSE), pLC_{20}, pLC_{50}, pLC_{80}, and iSPANs of EUG and 25 Mixture Rays at Three Exposure Volumes (EVs)

mixture ray/chemical	EV (μL)	α	β	RMSE	R^2	pLC_{20}	pLC_{50}	pLC_{80}	iSPAN
EUG	100	53.82	21.37	0.0361	0.9603	2.589	2.536	2.496	10.817
	200	50.40	19.44	0.0360	0.9884	2.670	2.611	2.568	9.839
	400	62.70	24.63	0.0215	0.9930	2.606	2.560	2.526	12.466
BEA-CID-EUG-R1	100	37.26	19.06	0.0437	0.9897	2.034	1.974	1.930	9.646
	200	35.21	17.92	0.0244	0.9963	2.049	1.985	1.938	9.069
	400	30.36	15.17	0.0396	0.9873	2.100	2.025	1.970	7.678
BEA-CID-EUG-R2	100	38.78	20.06	0.0359	0.9921	2.008	1.951	1.909	10.153
	200	35.02	18.16	0.0273	0.9949	2.011	1.949	1.902	9.191
	400	35.36	18.25	0.0507	0.9842	2.020	1.958	1.911	9.237
BEA-CID-EUG-R3	100	37.27	19.92	0.0469	0.9879	1.946	1.889	1.847	10.082
	200	28.45	15.02	0.0455	0.9845	1.994	1.919	1.862	7.602
	400	42.76	22.49	0.0339	0.9932	1.968	1.918	1.880	11.383
BEA-CID-EUG-R4	100	26.85	14.38	0.0598	0.9732	1.971	1.893	1.834	7.278
	200	35.73	18.68	0.0265	0.9956	1.993	1.932	1.887	9.454
	400	41.38	21.90	0.0420	0.9894	1.958	1.906	1.868	11.084
BEA-CID-EUG-R5	100	26.89	14.30	0.0514	0.9823	1.985	1.906	1.847	7.237
	200	31.58	16.61	0.0377	0.9905	1.992	1.923	1.873	8.406
	400	30.64	16.08	0.0366	0.9907	1.999	1.928	1.876	8.139
BEA-PHA-EUG-R1	100	36.53	20.35	0.0259	0.9965	1.869	1.813	1.772	10.300
	200	30.87	16.97	0.0442	0.9912	1.907	1.841	1.791	8.589
	400	49.74	27.27	0.0291	0.9962	1.879	1.837	1.807	13.801
BEA-PHA-EUG-R2	100	32.27	18.38	0.0307	0.9952	1.837	1.776	1.730	9.302
	200	31.81	17.62	0.0420	0.9925	1.890	1.826	1.778	8.918
	400	40.33	21.80	0.0300	0.9959	1.919	1.867	1.828	11.034
BEA-PHA-EUG-R3	100	28.09	15.15	0.0597	0.9817	1.953	1.878	1.823	7.668
	200	31.71	17.62	0.0606	0.9825	1.885	1.820	1.773	8.917
	400	35.47	19.08	0.0368	0.9938	1.938	1.878	1.834	9.656
BEA-PHA-EUG-R4	100	33.12	18.62	0.0521	0.9872	1.859	1.798	1.753	9.423
	200	29.22	16.58	0.0305	0.9950	1.853	1.784	1.734	8.391
	400	42.68	23.67	0.0338	0.9954	1.867	1.819	1.783	11.979
BEA-PHA-EUG-R5	100	29.74	16.50	0.0259	0.9968	1.893	1.825	1.774	8.351
	200	33.47	18.79	0.0435	0.9916	1.861	1.801	1.756	9.510
	400	28.07	15.37	0.0351	0.9941	1.924	1.850	1.795	7.779
PHA-CID-EUG-R1	100	32.50	16.61	0.0276	0.9958	2.047	1.979	1.928	8.406
	200	36.68	19.07	0.0279	0.9957	2.002	1.943	1.898	9.653
	400	35.98	17.69	0.0191	0.9981	2.119	2.055	2.007	8.953
PHA-CID-EUG-R2	100	31.98	16.66	0.0207	0.9974	2.010	1.942	1.891	8.432
	200	32.32	16.72	0.0305	0.9951	2.023	1.955	1.905	8.462
	400	42.13	20.74	0.0374	0.9936	2.104	2.049	2.008	10.498
PHA-CID-EUG-R3	100	34.18	17.99	0.0240	0.9969	1.983	1.920	1.873	9.105
	200	29.01	15.16	0.0301	0.9950	2.013	1.938	1.882	7.673
	400	35.93	18.15	0.0245	0.9970	2.062	2.000	1.953	9.185
PHA-CID-EUG-R4	100	33.79	17.37	0.0493	0.9876	2.032	1.966	1.918	8.791
	200	30.04	15.12	0.0255	0.9965	2.086	2.011	1.955	7.653
	400	45.67	22.84	0.0324	0.9953	2.065	2.016	1.979	11.561
PHA-CID-EUG-R5	100	30.25	15.43	0.0298	0.9955	2.058	1.984	1.930	7.809
	200	32.41	16.03	0.0205	0.9980	2.115	2.045	1.992	8.114
	400	48.30	23.79	0.0346	0.9944	2.093	2.046	2.010	12.041
BEA-PHA-CID-R1	100	29.54	15.27	0.0296	0.9956	2.033	1.959	1.903	7.729
	200	17.35	8.61	0.0365	0.9927	2.189	2.058	1.960	4.358
	400	20.75	11.14	0.0413	0.9838	1.997	1.896	1.820	5.638
BEA-PHA-CID-R2	100	25.10	12.85	0.0455	0.9875	2.070	1.982	1.916	6.504
	200	21.83	10.97	0.0313	0.9948	2.127	2.023	1.947	5.552
	400	19.59	9.94	0.0461	0.9868	2.122	2.008	1.923	5.031
BEA-PHA-CID-R3	100	31.91	17.39	0.0491	0.9844	1.921	1.856	1.808	8.801
	200	41.04	22.97	0.0791	0.9449	1.852	1.803	1.766	11.625
	400	42.95	23.55	0.0263	0.9959	1.887	1.839	1.804	11.919
BEA-PHA-CID-R4	100	24.14	13.51	0.0428	0.9779	1.898	1.814	1.752	6.838
	200	23.41	12.93	0.0349	0.9802	1.927	1.839	1.774	6.544
	400	28.04	15.72	0.0143	0.9978	1.879	1.807	1.753	7.956

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249

Table 1. continued

mixture ray/chemical	EV (μL)	α	β	RMSE	R^2	pLC_{20}	pLC_{50}	pLC_{80}	iSPAN
BEA-PHA-CID-R5	100	35.94	20.06	0.0194	0.9964	1.866	1.810	1.768	10.152
	200	22.70	12.30	0.0380	0.9897	1.967	1.875	1.807	6.225
	400	33.93	18.93	0.0173	0.9970	1.872	1.812	1.767	9.580
BEA-PHA-CID-EUG-R1	100	45.74	25.36	0.0361	0.9917	1.863	1.818	1.785	12.835
	200	38.17	21.00	0.0487	0.9856	1.889	1.835	1.795	10.629
	400	60.00	31.93	0.0356	0.9951	1.926	1.891	1.864	16.160
BEA-PHA-CID-EUG-R2	100	37.68	19.92	0.0522	0.9812	1.967	1.910	1.868	10.083
	200	33.98	19.03	0.0480	0.9860	1.864	1.805	1.761	9.631
	400	41.29	21.80	0.0513	0.9817	1.963	1.911	1.872	11.034
BEA-PHA-CID-EUG-R3	100	36.82	20.07	0.0377	0.9919	1.909	1.853	1.811	10.158
	200	52.95	29.35	0.0299	0.9953	1.855	1.817	1.788	14.854
	400	38.52	21.01	0.0365	0.9923	1.905	1.851	1.811	10.634
BEA-PHA-CID-EUG-R4	100	28.57	15.60	0.0554	0.9772	1.928	1.855	1.801	7.895
	200	55.68	31.38	0.0466	0.9884	1.822	1.786	1.759	15.881
	400	30.02	16.41	0.0434	0.9869	1.921	1.852	1.800	8.305
BEA-PHA-CID-EUG-R5	100	26.66	14.45	0.0430	0.9903	1.949	1.870	1.812	7.314
	200	23.95	12.75	0.0357	0.9923	1.996	1.907	1.841	6.453
	400	31.03	16.38	0.0439	0.9907	1.986	1.917	1.865	8.290

treatment of dental conditions such as pulp disease. EUG is commonly used as a painkiller to relieve pain during pulpitis.[10] In addition, EUG has been reported to have multiple biological effects, including antiviral, antioxidant, and anti-inflammatory effects.[11,12] The antibacterial properties of EUG suggest that it may interfere with natural fouling succession, and it degrades in the environment through photolysis and biodegradation.[13] Multiple evidence suggest that EUG may be effective in cancer prevention and chemotherapy, as well as inducing apoptosis and acting as an anticancer agent in several tumor types, inhibiting the viability of lung cancer cells.[10] Some scholars have pointed out that EUG also has significant periapical toxicity.[14] Previous studies have reported that EUG is cytotoxic to mouse fibroblast cell line L929, rat liver cells, dental pulp cells, and oral mucosa fibroblasts in vitro.[15−18] In addition, EUG may also damage rat oral mucosa in vivo.[19] While EUG is closely related to human life, excessive use may have a certain impact on human health and the environment. In particular, the use of EUG may alleviate some adverse reactions in organisms.[20−25] Therefore, systematic toxicological studies are needed to determine whether the addition of EUG will affect the toxicity and toxicological interaction of the flavor mixture.

For a mixture, due to the different mixing ratios of each component, there are countless rays of the mixture formed, so it is impossible to analyze all of the rays one by one. In this case, it is necessary to select representative multiple rays of the mixture for experimental study.[1] The uniform design ray (UD-Ray) method developed by our laboratory is an effective method to analyze the combined toxicity of mixtures with three or more components.[26,27]

In this study, four flavor chemicals BEA, PHA, CID, and EUG were selected as the target compounds. Four ternary and one quaternary mixture systems were constructed from these flavor compounds. Five representative mixture rays were designed for each mixture system using the UD-Ray method. Through experimental studies for determining the lethal toxicity of all four flavor compounds and mixture rays at three different exposure volumes on *C. elegans*, the nonlinear least-squares method was used for the concentration−response (lethality) curve (CRC) fitting, using iSPAN for quantitative characterization of toxicity sensitivity of various rays; the combination index (CI) was used to assess the toxicological interactions and then draw heatmaps. The combined toxicities and toxicity sensitivities of the flavor mixtures were evaluated, and the effects of the addition of EUG on flavor mixtures were analyzed from the perspective of toxicological interaction and toxicity sensitivity combined with previous studies, providing more references for the study of EUG and other flavor chemicals and their mixtures.

2. RESULTS AND DISCUSSION

2.1. Toxicity and iSPAN of Single Compounds. Figure 1 shows the CRCs of the 24 h mortality of EUG to *C. elegans* at three different exposure volumes. It can be seen from the figure that the dose−effect relationship at three exposure volumes can be effectively fitted by the nonlinear Weibull function. The fitting parameters (location α and shape β) and goodness-of-fit (determinant coefficient R^2 and root-mean-square error RMSE) are listed in Table 1. From the goodness-of-fit, it can be seen that EUG has significant concentration-dependent toxicity to *C. elegans*, which is the same as EUG's cytotoxicity.[28] It has also been found that EUG may reduce dehydrogenase activity in human osteoblasts in a concentration-dependent manner.[14] However, studies have shown that EUG leads to an increase in aspartate aminotransferase, alanine aminotransferase, and total bilirubin levels, and this effect does not seem to be concentration-dependent.[29] The three fitting CRCs do not completely coincide. The position of CRC at 200 μL is higher than the other two, and its confidence interval does not overlap with them, indicating that the exposure volume has a significant impact on the toxicity of EUG. The mean ± 2 times standard deviation of pLC_{50} of EUG is 2.569 ± 0.077. Compared with CID (3.130 ± 0.032), the toxicity of EUG is lower than that of CID; exposure volume has a greater effect on toxicity, while has a smaller effect than that of BEA (1.613 ± 0.171) (Table S1). However, EUG is more toxic than BEA and is close to some pesticides.[27,30]

The pLC_{20} and pLC_{80} at three exposure volumes are obtained through the CRCs; the iSPANs are calculated and listed in Table 1. The mean of iSPANs of the three exposed volumes ± 2 times standard deviation is expressed as 11.041 ±

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249

Table 2. Basic Concentration Compositions (BCCs) of 25 Rays in Five Mixture Systems and Mixture Ratios (p) of Various Components Calculated from the BCCs

mixture ray	BCC_{BEA}	BCC_{PHA}	BCC_{CID}	BCC_{EUG}	p_{BEA}	p_{PHA}	p_{CID}	p_{EUG}
BEA-CID-EUG-R1	EC_{10}		EC_{20}	EC_{30}	0.8489		0.0321	0.1190
BEA-CID-EUG-R2	EC_{20}		EC_{40}	EC_{10}	0.8793		0.0315	0.0892
BEA-CID-EUG-R3	EC_{30}		EC_{10}	EC_{40}	0.8814		0.0222	0.0963
BEA-CID-EUG-R4	EC_{40}		EC_{30}	EC_{20}	0.8938		0.0249	0.0813
BEA-CID-EUG-R5	EC_{50}		EC_{50}	EC_{50}	0.8887		0.0254	0.0859
BEA-PHA-EUG-R1	EC_{10}	EC_{20}		EC_{30}	0.4661	0.4686		0.0653
BEA-PHA-EUG-R2	EC_{20}	EC_{40}		EC_{10}	0.5021	0.4469		0.0509
BEA-PHA-EUG-R3	EC_{30}	EC_{10}		EC_{40}	0.5488	0.3912		0.0600
BEA-PHA-EUG-R4	EC_{40}	EC_{30}		EC_{20}	0.5546	0.3950		0.0504
BEA-PHA-EUG-R5	EC_{50}	EC_{50}		EC_{50}	0.5587	0.3873		0.0540
PHA-CID-EUG-R1		EC_{10}	EC_{20}	EC_{30}		0.8428	0.0334	0.1238
PHA-CID-EUG-R2		EC_{20}	EC_{40}	EC_{10}		0.8596	0.0367	0.1037
PHA-CID-EUG-R3		EC_{30}	EC_{10}	EC_{40}		0.8523	0.0277	0.1200
PHA-CID-EUG-R4		EC_{40}	EC_{30}	EC_{20}		0.8601	0.0328	0.1071
PHA-CID-EUG-R5		EC_{50}	EC_{50}	EC_{50}		0.8470	0.0350	0.1181
BEA-PHA-CID-R1	EC_{10}	EC_{30}	EC_{20}		0.4816	0.5002	0.0182	
BEA-PHA-CID-R2	EC_{20}	EC_{10}	EC_{40}		0.5455	0.4350	0.0195	
BEA-PHA-CID-R3	EC_{30}	EC_{40}	EC_{10}		0.5492	0.4370	0.0139	
BEA-PHA-CID-R4	EC_{40}	EC_{20}	EC_{30}		0.5824	0.4014	0.0162	
BEA-PHA-CID-R5	EC_{50}	EC_{50}	EC_{50}		0.5807	0.4026	0.0166	
BEA-PHA-CID-EUG-R1	EC_{10}	EC_{20}	EC_{30}	EC_{40}	0.4561	0.4586	0.0185	0.0667
BEA-PHA-CID-EUG-R2	EC_{20}	EC_{40}	EC_{10}	EC_{30}	0.4913	0.4373	0.0139	0.0576
BEA-PHA-CID-EUG-R3	EC_{30}	EC_{10}	EC_{40}	EC_{20}	0.5423	0.3866	0.0174	0.0537
BEA-PHA-CID-EUG-R4	EC_{40}	EC_{30}	EC_{20}	EC_{10}	0.5491	0.3910	0.0142	0.0457
BEA-PHA-CID-EUG-R5	EC_{50}	EC_{50}	EC_{50}	EC_{50}	0.5499	0.3812	0.0157	0.0532

2.655. Compared with BEA (4.222 ± 1.430) and CID (8.594 ± 1.934), EUG has a larger iSPAN. However, it is smaller than PHA (20.055 ± 9.748), and it can be seen from the standard deviation that the exposure volume has a certain effect on iSPAN of EUG, but it is much smaller than that of PHA (Table S1). Compared with other substances such as cadmium chloride, copper chloride, zinc chloride, gallic acid, and nonylphenol ethoxide,[31−33] the iSPAN of EUG is much larger. In conclusion, the flavor chemicals have a significant toxic effect on *C. elegans* and also have high toxicity sensitivity, and both of them change with the change in the exposure volume. However, whether this is a unique property of the flavor chemicals remains to be investigated, and a large number of other flavor chemicals need to be evaluated before they can be verified. Therefore, when the concentration of the substance with large iSPAN changes slightly, the toxicity changes significantly, and the toxicity is also affected by the exposure volume. Therefore, the dosage and the treatment process of these substances, such as flavor chemicals in production and life, need to be strictly controlled. Otherwise, their indiscriminate discharge together with other substances will cause serious harm to organisms or the ecological environment. In the toxicity assessment of these substances, it is necessary to reduce the change rate of the concentration to obtain more effective concentrations, and the influence of exposure volume should be considered.

2.2. Change of Combined Toxicities and iSPANs of Mixture Rays. The UD-Ray method was used to design five mixture rays with different mixing ratios for each ternary and quaternary mixture system, respectively. Table 2 lists the basic concentration composition of each ray in the five mixture systems, the mixing ratio of each component in the mixture, and its CRC fitting parameters (location α and shape β) and goodness-of-fit (determination coefficient R^2 and root-mean-square error RMSE) are listed in Table 1. The dose−effect relationship of the 15 rays of BEA-CID-EUG, BEA-PHA-EUG, and PHA-CID-EUG ternary mixture systems that contain EUG at three different exposure volumes is shown in Figure 1, and that of rays of the ternary mixture system BEA-PHA-CID, that without EUG, and a quaternary mixture system BEA-PHA-CID-EUG that contains EUG is shown in Figure 2. It can be seen from Figures 1 and 2 that all of the mixture rays have significant toxic effects on *C. elegans*, while the toxicity increases with the increase in the concentration of the mixture, and all of them can be effectively fitted by the two-parameter Weibull model. The CRCs of the five rays in each ternary mixture system in Figures 1 and 2 have a similar shape and inclination degree as a whole. Except for rays of PHA-CID-EUG and BEA-PHA-CID-EUG systems, the CRCs of other mixture rays almost overlapped, indicating that it is not only for the binary flavor mixture rays,[1] a slight change in the exposure volume also affects the combined toxicity of the ternary and quaternary flavor mixture rays.

Table 1 lists the pLC_{50} and iSPAN of each ray in each mixture system at three exposure volumes. For the BEA-CID-EUG system, the toxicities of five rays at three different exposure volumes are expressed using 3 pLC_{50} mean ± 2 times standard deviation as 1.995 ± 0.054 (BEA-CID-EUG-R1), 1.953 ± 0.009 (BEA-CID-EUG-R2), 1.909 ± 0.034 (BEA-CID-EUG-R3), 1.910 ± 0.040 (BEA-CID-EUG-R4), and 1.919 ± 0.023 (BEA-CID-EUG-R5). According to the standard deviation, it can be found that the exposure volume has no significant influence on the combined toxicity. By comparing the standard deviations of toxicity of BEA-CID-R1 (0.229) and BEA-CID-R2 (0.214) (Table S2) in the BEA-CID system without EUG, after the addition of EUG, the influence

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249

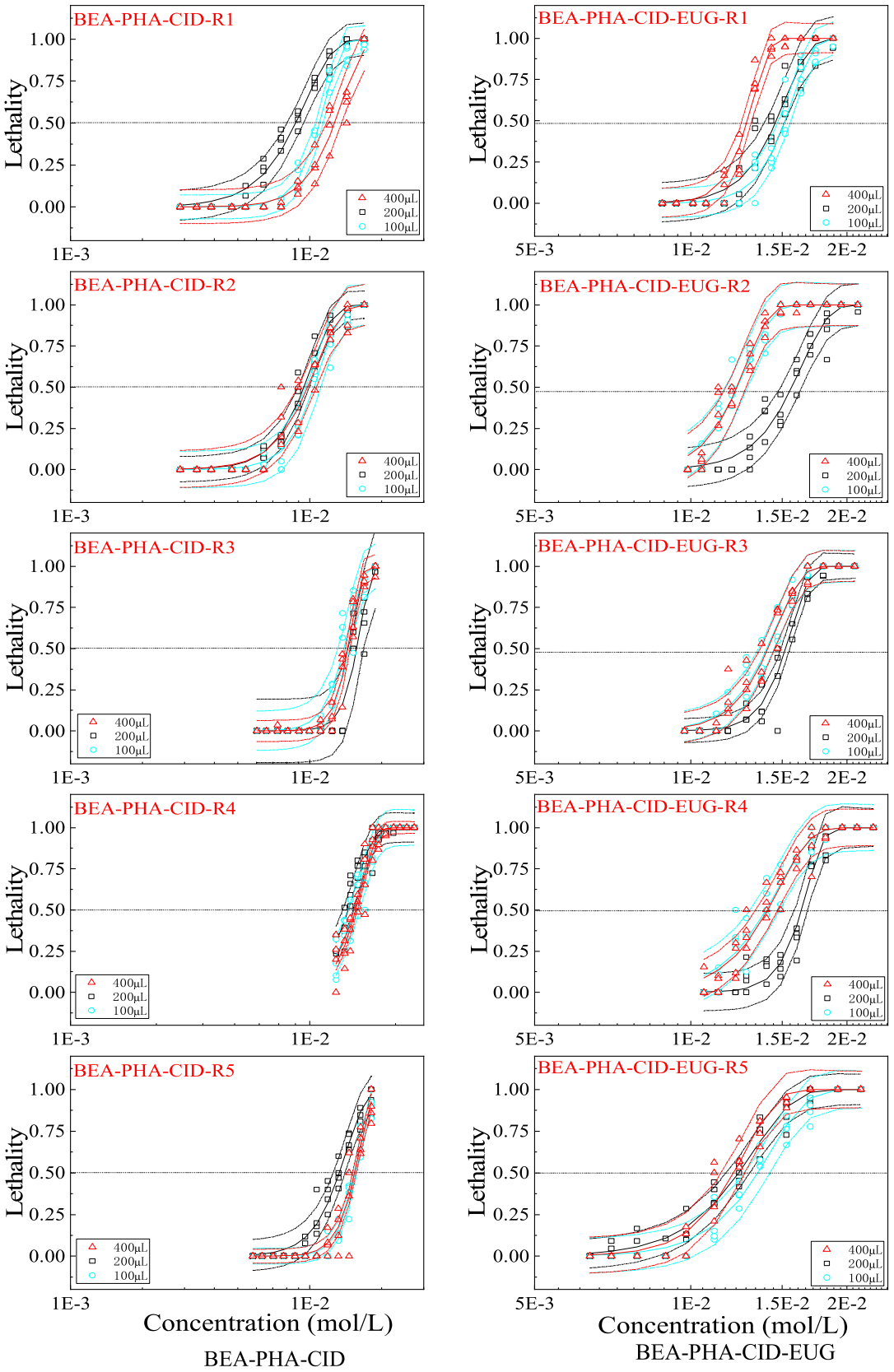


Figure 2. Concentration−response curves of 10 mixture rays in BEA-PHA-CID and BEA-PHA-CID-EUG systems at three exposure volumes, where — refers to the fitting curves and ··· refers to the 95% observation-based confidence intervals (OCIs).

of the change in the exposed volume on the toxicity of the mixture is weakened. The iSPANs of five rays at three different exposure volumes are expressed using iSPAN mean ± 2 times standard deviation as 8.798 ± 2.023 (BEA-CID-EUG-R1), 9.527 ± 1.085 (BEA-CID-EUG-R2), 9.689 ± 3.842 (BEA-CID-EUG-R3), 9.272 ± 3.819 (BEA-CID-EUG-R4), and

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249

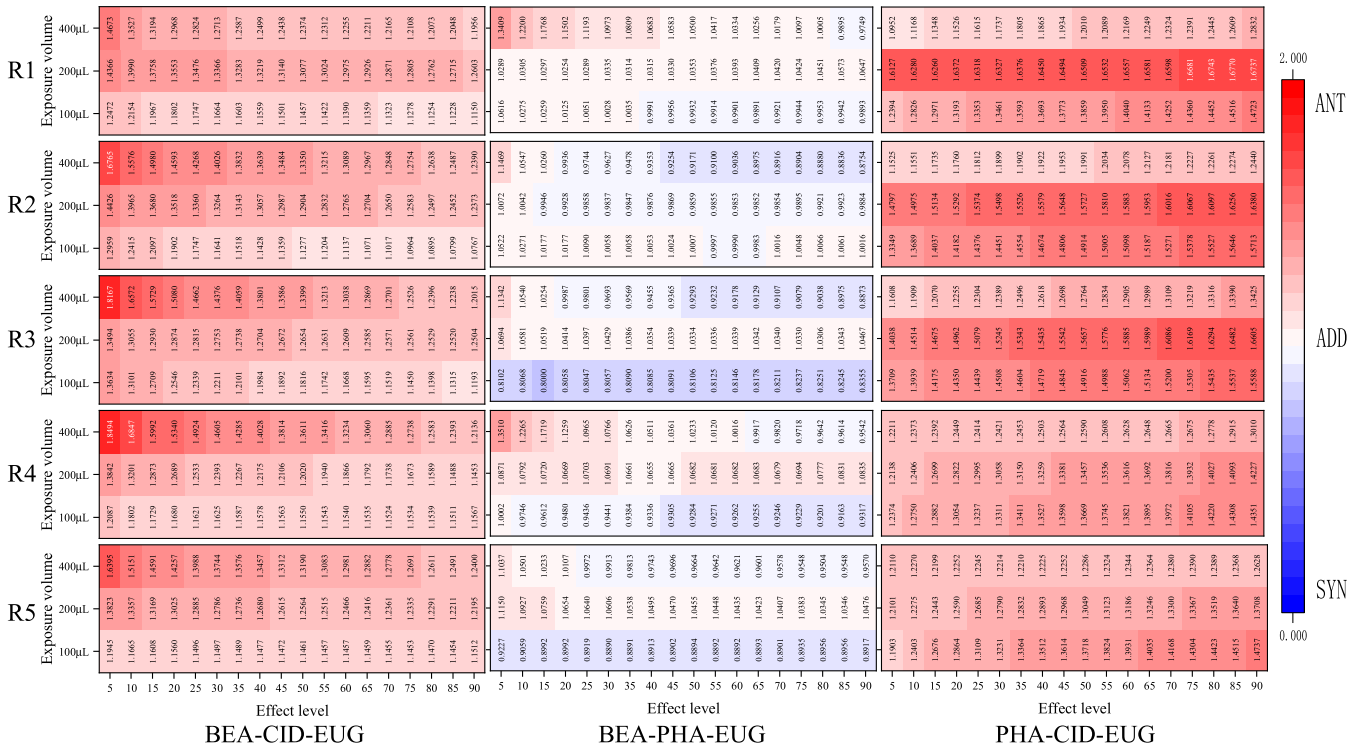


Figure 3. CI heatmaps of BEA-CID-EUG, BEA-PHA-EUG, and PHA-CID-EUG systems at three exposure volumes of 100, 200, and 400 μL, where blue, white, and red colors refer to synergistic interaction (SYN), additive action (ADD), and antagonistic interaction (ANT), respectively. Here, the deeper the color, the stronger the interaction.

7.927 ± 1.225 (BEA-CID-EUG-R5). The iSPAN of BEA-CID-EUG-R3 is the largest, and BEA-CID-EUG-R5 has the smallest iSPAN. According to the 2 times standard deviation, the exposure volume has the least influence on the iSPAN of BEA-CID-EUG-R2. Compared with the BEA-CID system, the addition of EUG increases the iSPAN of the mixture system (Table S2), and the exposure volume has a more significant influence on it.

For the BEA-PHA-EUG system, the toxicities of five rays are 1.830 ± 0.030 (BEA-PHA-EUG-R1), 1.823 ± 0.091 (BEA-PHA-EUG-R2), 1.859 ± 0.067 (BEA-PHA-EUG-R3), 1.800 ± 0.035 (BEA-PHA-EUG-R4), and 1.825 ± 0.049 (BEA-PHA-EUG-R5); the five means are close, and the 2 times standard deviation values are all less than 0.100. It can be seen that the exposure volume has little influence on the toxicity of the system, and the toxicity of the three CRCs under each mixing ratio has little change. The toxicity of the system is slightly higher than that of the BEA-PHA system, and the effect of the exposure volume on the toxicity of each ray is not significant as in the BEA-PHA system.[1] The iSPANs of the five rays are 10.897 ± 5.313 (BEA-PHA-EUG-R1), 9.751 ± 2.254 (BEA-PHA-EUG-R2), 8.747 ± 2.010 (BEA-PHA-EUG-R3), 9.931 ± 3.694 (BEA-PHA-EUG-R4), and 8.547 ± 1.764 (BEA-PHA-EUG-R5). The change in the exposure volume has significant and varying degrees of influence on iSPAN of the system. By comparing the 2 standard deviation values, it can be found that BEA-PHA-EUG-R1 has the most significant influence. The largest iSPAN is BEA-PHA-EUG-R1. However, compared with BEA-PHA-R5 (19.519 ± 11.289) (Table S2), the iSPANs of the BEA-PHA-EUG system are much smaller, and the exposure volume has less influence on it.

For the PHA-CID-EUG system, the toxicities of the five rays are 1.992 ± 0.114 (PHA-CID-EUG-R1), 1.982 ± 0.117 (PHA-CID-EUG-R2), 1.953 ± 0.084 (PHA-CID-EUG-R3), 1.998 ± 0.055 (PHA-CID-EUG-R4), and 2.025 ± 0.071 (PHA-CID-EUG-R5); the five means are close, indicating that the toxicity of the system is relatively stable. The 2 times standard deviation of PHA-CID-EUG-R4 is low, so the exposure volume had no significant effect on its toxicity, but it had a significant effect on the toxicity of the other four rays. Compared with the PHA-CID system without EUG, the toxicity of the PHA-CID-EUG system is close to it (Table S2). The iSPANs of the five rays are 9.004 ± 1.250 (PHA-CID-EUG-R1), 9.131 ± 2.368 (PHA-CID-EUG-R2), 8.654 ± 1.702 (PHA-CID-EUG-R3), 9.335 ± 4.020 (PHA-CID-EUG-R4), and 9.321 ± 4.720 (PHA-CID-EUG-R5). The iSPAN of each ray in the system is close, and the change in the exposure volume has a certain influence on the iSPAN of the system, but the influence degree is not the same. By comparing the 2 times standard deviation value, it can be found that the influence on PHA-CID-EUG-R4 and PHA-CID-EUG-R5 is the most significant. However, the effect is slightly smaller than that of PHA-CID-R1 (12.198 ± 7.900) and PHA-CID-R2 (8.673 ± 6.102) (Table S2), indicating that the overall effect of the exposure volume on iSPAN in a ternary mixture system is weakened by the addition of EUG.

For the BEA-PHA-CID system, the toxicities of the five rays are 1.971 ± 0.163 (BEA-PHA-CID-R1), 2.004 ± 0.041 (BEA-PHA-CID-R2), 1.833 ± 0.054 (BEA-PHA-CID-R3), 1.820 ± 0.034 (BEA-PHA-CID-R4), and 1.832 ± 0.074 (BEA-PHA-CID-R5). The toxicities of the five rays are similar, except for BEA-PHA-CID-R1 and BEA-PHA-CID-R5. The exposure volume has no significant effect on the toxicities of the other rays in the BEA-PHA-CID system. The iSPANs of the five rays are 5.908 ± 3.403 (BEA-PHA-CID-R1), 5.696 ± 1.494 (BEA-PHA-CID-R2), 10.782 ± 3.443 (BEA-PHA-CID-R3), 7.113 ±

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249

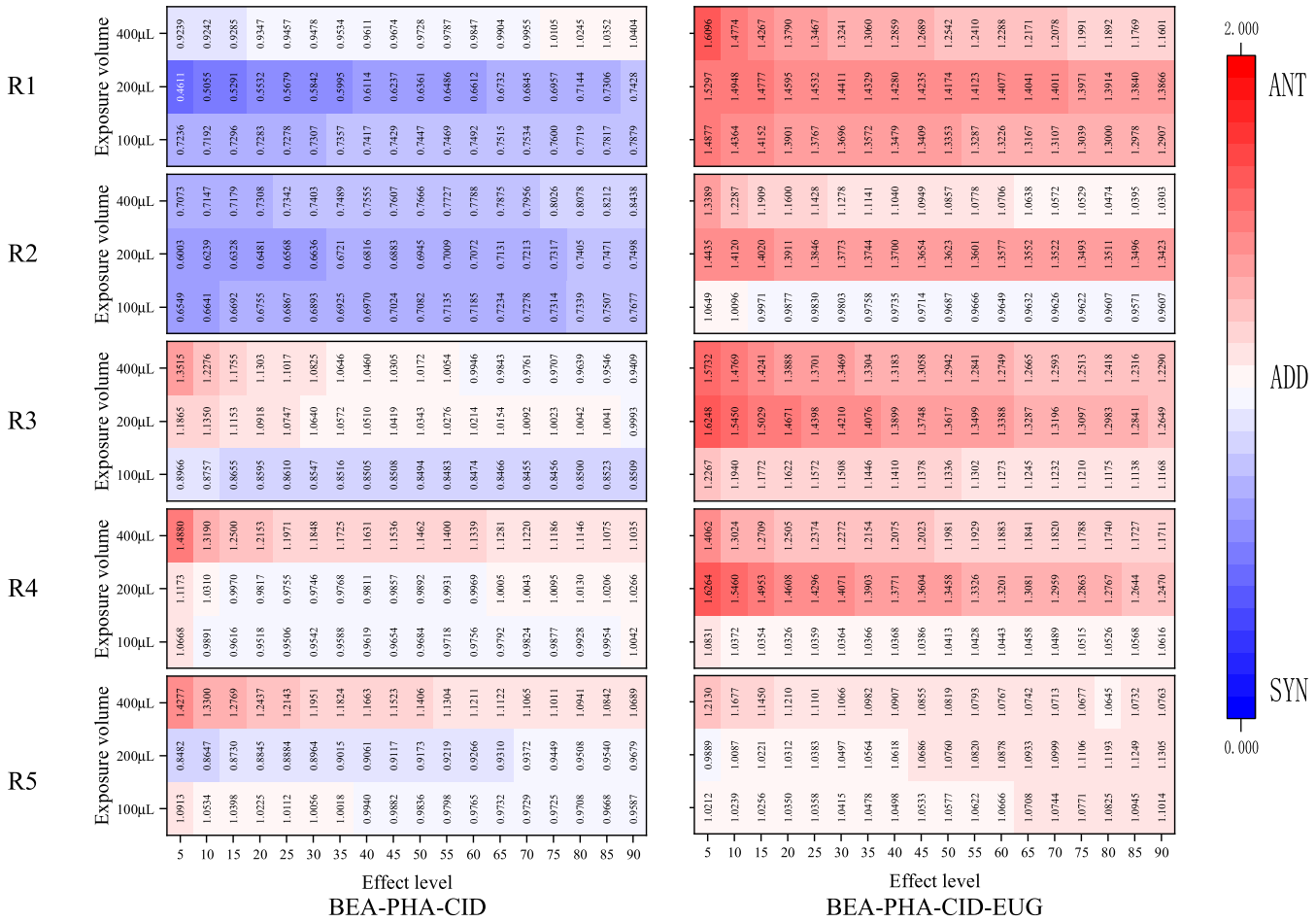


Figure 4. CI heatmaps of BEA-PHA-CID and BEA-PHA-CID-EUG systems at three exposure volumes of 100, 200, and 400 μL, where blue, white, and red colors refer to synergistic interaction (SYN), additive action (ADD), and antagonistic interaction (ANT), respectively. Here, the deeper the color, the stronger the interaction.

1.490 (BEA-PHA-CID-R4), and 8.652 ± 4.243 (BEA-PHA-CID-R5). It can be seen that the change in the exposure volume has a certain effect on the iSPAN of the system, among which BEA-PHA-CID-R1, BEA-PHA-CID-R3, and BEA-PHA-CID-R5 are more significant.

For the BEA-PHA-CID-EUG system, the toxicities of the five rays are 1.848 ± 0.076 (BEA-PHA-CID-EUG-R1), 1.875 ± 0.122 (BEA-PHA-CID-EUG-R2), 1.840 ± 0.040 (BEA-PHA-CID-EUG-R3), 1.831 ± 0.078 (BEA-PHA-CID-EUG-R4), and 1.898 ± 0.050 (BEA-PHA-CID-EUG-R5). The toxicity of the system is relatively stable, which is close to the BEA-PHA-CID system without EUG. At the same time, the exposure volume has a significant impact on BEA-PHA-CID-EUG-R1, BEA-PHA-CID-EUG-R2, and BEA-PHA-CID-EUG-R4. The iSPANs of the five rays are 13.208 ± 5.569 (BEA-PHA-CID-EUG-R1), 10.249 ± 1.432 (BEA-PHA-CID-EUG-R2), 11.882 ± 5.170 (BEA-PHA-CID-EUG-R3), 10.694 ± 8.994 (BEA-PHA-CID-EUG-R4), and 7.352 ± 1.838 (BEA-PHA-CID-EUG-R5). The change in the exposure volume also has certain and varying degrees of influence on the iSPAN of the system. By comparing the 2 times standard deviation value, it can be found that the influence of the exposure volume on BEA-PHA-CID-EUG-R1, BEA-PHA-CID-EUG-R3, and BEA-PHA-CID-EUG-R4 is more significant. Among them, BEA-PHA-CID-EUG-R4 has the largest influence. Compared with the BEA-PHA-CID system, iSPAN tended to increase, while the exposure volume also had a stronger effect on iSPAN.

In conclusion, not only the rays of binary mixtures of flavors but also the rays of ternary and quaternary mixture systems still have a significant toxicity effect on *C. elegans* and larger iSPAN, and the toxicity and iSPAN are also affected by the exposure volume to varying degrees. Moreover, the influence on the toxicity of the mixture rays with the change in the exposure volume still exists after the addition of EUG. For iSPAN, the addition of EUG changes the iSPAN of the original mixture system, and the change in the exposure volume has different effects on iSPAN.

2.3. Effect on Toxicological Interactions of the Mixtures. Figures 3 and 4 show the toxicological interaction heatmaps of five mixture systems. The abscissa represents the effect, and the values in the heatmaps represent the CI value under the effect. Blue, white, and red colors represent synergistic interaction (SYN), additive action (ADD), and antagonistic interaction (ANT), respectively. The depth of the color directly reflects the strength of interaction. Our previous study indicated that for the BEA-CID system, five rays showed different toxicological interactions due to different exposure volumes, and most of them showed SYN. The strongest ANT in this system was BEA-CID-R4 at 400 μL, and its CI value was 1.3344 (Figure S1). However, after the addition of EUG, the overall heatmap shows red, that is, ANT. The toxicological

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249

interaction of each ray is similar, and the ANT is strong at low concentrations, and the maximum intensity of ANT increased with the increase in the exposure volume. The strongest ANT is BEA-CID-EUG-R4 at 400 μL. Its CI value is 1.8494. The ANT intensity of each ray decreased with the increase in the concentration at three exposure volumes, but there was no SYN.

The toxicological interaction of the BEA-PHA-EUG system is different from that of the BEA-CID-EUG system. There are more SYN in the mixture, and for the same ray, the change in the exposure volume has a significant effect on the interaction under the condition of a constant concentration. The interactions of BEA-PHA-EUG and BEA-PHA systems do not show the same significant change as BEA-CID-EUG and BEA-CID systems, but the maximum value of CI of ANT in the mixture decreased due to the addition of EUG, from 1.6897 in BEA-PHA-R2 at 400 μL (Figure S1) to 1.3510 in BEA-PHA-EUG-R4 at 400 μL.

The heatmap of the interaction of the PHA-CID-EUG system is similar to that of the BEA-CID-EUG system. The overall heatmap is red, which means that the mixture shows obvious ANT, and the color is deep, indicating strong ANT. The maximum CI value in the mixture is 1.6770, PHA-CID-EUG-R1 at 200 μL. In any exposed volume, the interaction of the five rays has the same change law, that is, the intensity of ANT increases with the increase in the concentration, which is contrary to the BEA-CID-EUG system. For the same mixing ratio, it can be seen from the color of the heatmap that the interaction will change with different exposed volumes. Although they all show ANT, the intensity is different. For the PHA-CID system (Figure S1), PHA-CID-R5 showed a deep blue color, indicating strong SYN. The other four rays showed ADD or even ANT at three exposed volumes. However, the ANT intensity was lower than that of the ternary mixture PHA-CID-EUG on the whole.

BEA-PHA-CID-R1 and BEA-PHA-CID-R2 in the BEA-PHA-CID system show strong SYN, with the minimum CI 0.4611 (BEA-PHA-CID-R1 at 200 μL), which is the strongest SYN of all of the flavor mixtures involved in this paper (Figure S1). The change in the exposure volume also affected the interaction of each ray in the mixture, especially in BEA-PHA-CID-R3, BEA-PHA-CID-R4, and BEA-PHA-CID-R5. As can be seen from the heatmap, even at the same concentration, the interaction will also change due to the change in the exposure volume. After the addition of EUG, the interaction of the BEA-PHA-CID-EUG system changes obviously compared with the original system. The whole is mainly ANT. The red color in BEA-PHA-CID-EUG-R1, BEA-PHA-CID-EUG-R3, and BEA-PHA-CID-EUG-R4 is deeper, which means that the ANT is strong. Even if the exposure volume changes the interaction of the rays, there is no obvious SYN in the mixture.

It can be seen from the results that, first of all, as with binary mixtures, the change in the exposure volume will still affect the toxicological interactions of ternary and quaternary mixtures to varying degrees. Second, in addition to the BEA-PHA system, the other three mixture systems BEA-CID, PHA-CID, and BEA-PHA-CID have different intensities of SYN. After the addition of EUG, new mixtures do not show obvious SYN; on the contrary, mixtures show strong ANT; that is to say, the addition of EUG makes the SYN into ANT, which suggests that EUG attenuates the toxicological interactions with the organism of flavor mixtures. Studies indicate that EUG is also used in agricultural applications to protect food from microorganisms such as *Listeria monocytogenes* and lactic acid bacteria during storage and as an insecticide and fumigant;[23] at the same time, EUG can inhibit the growth of bacteria and inhibit the production of *Staphylococcus aureus* exotoxin, which can be used as a food additive.[34] Therefore, adding EUG into the flavor mixture can effectively reduce its harm to the organism and has a certain positive effect on the ecological environment and human health. Zhang et al. found that the mixture containing Pb showed antagonism, but the mixture without Pb showed synergism; so they concluded that Pb may be the key component causing antagonism in the mixture,[35] which is similar to the conclusion of this study. EUG may also be the key component causing the weakening of synergistic toxicological interactions in flavor mixtures. Similarly, Zhang et al. also used the UD-Ray to conduct experiments and pointed out that $[bmim]C_8H_{17}SO_4$ is the key component that causes the antagonism of the ionic liquids mixture, and concluded that the UD-Ray is an effective method for screening key components.[36] Fan et al. studied ternary and quaternary mixtures composed of insecticides, ionic liquids, and antibiotics and concluded that polymyxin B sulfate was the key component to induce time-dependent antagonism.[37] Kumar et al. mixed EUG with cadmium and orally treated rats, and the results showed that EUG treatment was very effective; it significantly reversed the cadmium-induced biochemical changes, almost similar to the control group. That is to say, EUG has a protective effect against cadmium-induced toxicity,[38] which is similar to the toxicological interaction attenuating effect of EUG found in this study. Other studies have found that for mice, the addition of EUG alleviated oxidative stress and acute lung toxicity induced by C_{60} exposure, indicating that EUG can avoid functional changes and reduce lung tissue damage, which may be caused by EUG's antioxidant potential through regulating the inflammatory process.[39] At the same time, it has been pointed out that EUG is a potential antibacterial compound against *Salmonella typhi* and can be used to prevent or treat *S. typhi* infection.[3] Lung cancer, the leading cause of cancer-related morbidity and mortality worldwide, remains a serious public health problem.[40] Studies have shown that EUG at low doses can significantly inhibit lung cancer cell viability and may be an excellent drug to prevent lung cancer growth and metastasis.[10] This conclusion is similar to the attenuating effect of EUG on toxicological interaction found in this paper. However, this study just added EUG into the flavor mixtures; whether other kinds of substances have the same effect needs to be further studied and discussed. Some studies showed that EUG combined with conventional antibiotics detected a synergistic effect on Gram-negative bacteria, and combined with vancomycin and β-lactam, bacterial membrane damage increased, indicating a synergistic effect, which may be caused by different drug targets.[41,42] It can be seen that EUG does not necessarily produce the same effect as flavor mixtures when mixed with other substances.

3. CONCLUSIONS

This study chose four kinds of common flavor chemicals BEA, CID, PHA, and EUG as the target compounds, designed four ternary and one quaternary flavor mixture systems, and used the UD-Ray to design five rays for each mixture, respectively. The lethal toxicities of each ray to *C. elegans* at three different exposure volumes were measured. The toxicity sensitivity of each ray was quantitatively characterized by iSPAN, the

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249

toxicological interactions of all mixtures were evaluated by CI, and then heatmaps were drawn. The results show that, first of all, not only the binary mixture of flavor rays but ternary and quaternary mixture rays also have a significant toxicity effect on *C. elegans* and higher toxicity sensitivity. That is to say, the combined toxicity would change significantly with the slight change in the concentration of the binary mixture rays. Therefore, large iSPAN can be regarded as one of the characteristics of flavor chemicals and their mixtures, and this is also related to the phenomenon that the change in the exposure volume can affect the combined toxicity and iSPAN. Second, combined with the results of iSPAN and the heatmap of interaction in our previous study, it can be found that the addition of EUG will change the combined toxicity and iSPAN of the original binary or ternary mixture system to different degrees. For interactions, except for the BEA-PHA system, the other two binary and one ternary mixture systems without EUG show SYN, but show ANT after the addition of EUG to form two ternary and one quaternary mixture systems and have high strength at individual concentrations. That is to say, to some extent, the addition of EUG weakened the interaction of the flavor mixtures with *C. elegans*. This is related to some biological functions of EUG, and the specific reasons need to be further studied. The different properties and activities of EUG are still not well understood and need to be further explored by more long-term biological studies in vivo and in vitro.

4. MATERIALS AND METHODS

4.1. Test Chemicals. BEA, CID, PHA, and EUG were all purchased from Macklin (China). The information about BEA, CID, and PHA can be found in Table S1. The stock solution concentration of EUG (97-53-0) is 2.5 g·L^{-1}, and the purity is 99.0%. All solutions were prepared with Milli-Q water and stored at 4 °C, and prepared for immediate use. All four substances were soluble in water, and no cosolvent was added. The solution was colorless and transparent and could be observed normally under a microscope.

4.2. Nematode Culture and Mortality Test. The wild-type strains (N2) of *C. elegans* used in the experiments and the *E. coli* OP50 used as its food were all from the Institute of Medicine, Tongji University. *E. coli* OP50 culture, nematode culture, age synchronization, blank and treatment group design, the test concentration, and the design of three exposure volumes were the same as described in the literature.[1,43−45]

4.3. Design of Mixtures and Concentration−Response Fitting. To reasonably and effectively select the representative mixture rays in the mixture system for analysis, this paper uses the UD-Ray method[46] to design five rays for each mixture system. For ternary mixtures, a uniform table U_5 (5^3) is used, where the subscript 5 represents the number of mixture rays (R1, R2, R3, R4, and R5), 5 refers to the number of concentration levels of various components (EC_{10}, EC_{20}, EC_{30}, EC_{40}, and EC_{50}), and the superscript 3 refers to the factor (component) number. For quaternary mixtures, a uniform table U_5 (5^4) is used, where the subscript 5 represents the number of mixture rays (R1, R2, R3, R4, and R5), 5 refers to the number of concentration levels of various components (EC_{10}, EC_{20}, EC_{30}, EC_{40}, and EC_{50}), and the superscript 4 refers to the factor (component) number.[30,47] Design details of the basic concentration composition and mixing ratio of each component of five rays in the mixture system are given in Table 2. Appropriate dilution factors were used to design 12 concentrations of each mixture ray for the experiment.[27]

The mortality data at different concentrations were obtained through experiments. The Weibull two-parameter (location α and shape β) nonlinear fitting function[48,49] was used to fit the concentration effect data. The Weibull function expression is shown as follows

$$f(x) = 1 - \exp(-\exp(\alpha + \beta \cdot \lg(x))) \quad (1)$$

where $f(x)$ is the lethality to *C. elegans* and x is the concentration of a single component or a mixture ray. The determination coefficient R^2 and root-mean-square error (RMSE) were used to describe the goodness of fitting, and the 95% observation-based confidence intervals (OCIs) could represent the uncertainty of the experimental observation and curve fitting.[44]

4.4. Toxicological Interaction Evaluation. The toxicological interaction of a mixture is evaluated by the combination index[30] (CI); the CI equation is as follows

$$\mathrm{CI} = \sum_{i=1}^{m} \frac{c_i}{\mathrm{EC}_{x,i}} \quad (2)$$

where m is the number of components in mixtures, $EC_{x,i}$ is the concentration of the ith component that induces the x% effect when applied individually, and c_i is the concentration of the ith component in the mixture when it induces the x% effect. When CI is less than 1, the mixture produces synergism (SYN), while when CI is greater than 1, the mixture produces antagonism (ANT).[1] Finally, CI values of all mixture rays under different effects are presented in the form of an interaction heatmap, which could more intuitively reflect the rules of toxicological interaction of mixtures.

4.5. Quantitative Assessment of Toxicity Sensitivity. The iSPAN is reflected by the inverse of the span between the negative logarithms of LC_{20} and LC_{80} of a compound or a mixture ray to the organism.

$$\mathrm{iSPAN} = \frac{1}{\mathrm{pLC}_{20} - \mathrm{pLC}_{80}} \quad (3)$$

where pLC_{20} and pLC_{80} are the negative logarithms of LC_{20} and LC_{80}. The iSPAN value is positively correlated with the toxicity sensitivity of the substance.[1]

All of the above calculations, including the test concentrations, automatic calibration, mixture design, concentration−response (lethality rate) curve fitting, CI, pLC_{20}, and pLC_{80}, were derived from the APTox (assessment and prediction for the toxicity of chemical mixtures) program developed in our laboratory.[45]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03577.

Experimental data of BEA, CID, and PHA; experimental data of binary mixture rays; and heatmap of binary mixtures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Shu-Shen Liu − *Key Laboratory of Yangtze River Water Environment, Ministry of Education, College of*

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249

Environmental Science and Engineering, Tongji University, Shanghai 200092, P. R. China; State Key Laboratory of Pollution Control and Resource Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, P. R. China; Shanghai Institute of Pollution Control and Ecological Security, Shanghai 200092, P. R. China; orcid.org/0000-0002-2160-9750; Phone: (86)-021-65982767; Email: ssliuhl@263.net

Authors

Sheng Lu − *Key Laboratory of Yangtze River Water Environment, Ministry of Education, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, P. R. China; Shanghai Institute of Pollution Control and Ecological Security, Shanghai 200092, P. R. China; CSCEC AECOM Consultants Co. Ltd., Lanzhou, Gansu 730000, P. R. China*

Peng Huang − *Key Laboratory of Yangtze River Water Environment, Ministry of Education, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, P. R. China; State Key Laboratory of Pollution Control and Resource Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, P. R. China*

Ze-Jun Wang − *Key Laboratory of Yangtze River Water Environment, Ministry of Education, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, P. R. China; Shanghai Institute of Pollution Control and Ecological Security, Shanghai 200092, P. R. China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c03577

Author Contributions

CRediT: S.L. designed the experiments and wrote the manuscript. S.-S.L. provided guidance in writing papers and designed the experiments. P.H. performed the experiments. Z.-J.W. analyzed the data.

Notes

The authors declare no competing financial interest.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

■ ACKNOWLEDGMENTS

The authors are thankful to the National Natural Science Foundation of China (21976139, 22176143) for their financial support.

■ REFERENCES

(1) Lu, S.; Liu, S.-S.; Huang, P.; Wang, Z.-J.; Wang, Y. Study on the combined toxicities and quantitative characterization of toxicity sensitivities of three flavor chemicals and their mixtures to *Caenorhabditis elegans*. *ACS Omega* **2021**, *6*, 35745−35756.

(2) Li, J.; Liu, X.; Liang, X.; Zhang, M.; Han, L.; Song, J. Simultaneous detection of five flavoring agents in chewing gum by ultrasound-microwave synergistic extraction coupled with gas chromatography. *Sci. Rep.* **2019**, *9*, No. 12085.

(3) Devi, K. P.; Nisha, S. A.; Sakthivel, R.; Pandian, S. K. Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. *J. Ethnopharmacol.* **2010**, *130*, 107−115.

(4) Gueretz, J. S.; Somensi, C. A.; Martins, M. L.; Souza, A. P. d. Evaluation of eugenol toxicity in bioassays with test-organisms. *Cienc. Rural* **2017**, *47*, No. e20170194.

(5) Cortés-Rojas, D. F.; de Souza, C. R. F.; Oliveira, W. P. Clove (Syzygium aromaticum): a precious spice. *Asian Pac. J. Trop. Biomed.* **2014**, *4*, 90−96.

(6) Pereira, R. B.; Pinto, N. F. S.; Fernandes, M. J. G.; Vieira, T. F.; Rodrigues, A. R. O.; Pereira, D. M.; Sousa, S. F.; Castanheira, E. M. S.; Fortes, A. G.; Goncalves, M. S. T. Amino alcohols from eugenol as potential semisynthetic insecticides: chemical, biological, and computational insights. *Molecules* **2021**, *26*, 6616.

(7) Fernandes, M. J. G.; Pereira, R. B.; Pereira, D. M.; Fortes, A. G.; Castanheira, E. M. S.; Goncalves, M. S. T. New eugenol derivatives with enhanced insecticidal activity. *Int. J. Mol. Sci.* **2020**, *21*, 9257.

(8) Al Wafai, R.; El-Rabih, W.; Katerji, M.; Safi, R.; El Sabban, M.; El-Rifai, O.; Usta, J. Chemosensitivity of MCF-7 cells to eugenol: release of cytochrome-c and lactate dehydrogenase. *Sci. Rep.* **2017**, *7*, No. 43730.

(9) Zari, A. T.; Zari, T. A.; Hakeem, K. R. Anticancer properties of eugenol: a review. *Molecules* **2021**, *26*, 7407.

(10) Fangjun, L.; Zhijia, Y. Tumor suppressive roles of eugenol in human lung cancer cells. *Thorac. Cancer* **2018**, *9*, 25−29.

(11) Waldman, S. A.; Hyslop, T.; Schulz, S.; Barkun, A.; Nielsen, K.; Haaf, J.; Bonaccorso, C.; Li, Y.; Weinberg, D. S. Association of GUCY2C expression in lymph nodes with time to recurrence and disease-free survival in pN0 colorectal cancer. *JAMA, J. Am. Med. Assoc.* **2009**, *301*, 745−752.

(12) Agustinisari, I.; Mulia, K.; Nasikin, M. The effect of eugenol and chitosan concentration on the encapsulation of eugenol using whey protein−maltodextrin conjugates. *Appl. Sci.* **2020**, *10*, 3205.

(13) García, M.; Stupak, M.; Pérez, M.; Blustein, G. Transitioning to nontoxic antifouling paints. *Pigm. Resin Technol.* **2015**, *44*, 116−121.

(14) Ho, Y. C.; Huang, F. M.; Chang, Y. C. Mechanisms of cytotoxicity of eugenol in human osteoblastic cells in vitro. *Int. Endod. J.* **2006**, *39*, 389−393.

(15) Hume, W. R. Effect of eugenol on respiration and division in human pulp, mouse fibroblasts, and liver-cells invitro. *J. Dent. Res.* **1984**, *63*, 1262−1265.

(16) Kasugai, S.; Hasegawa, N.; Ogura, H. Application of the MTT colorimetric assay to measure cytotoxic effects of phenolic compounds on established rat dental pulp cells. *J. Dent. Res.* **1991**, *70*, 127−130.

(17) Thompson, D. C.; Constantinteodosiu, D.; Moldeus, P. Metabolism and cytotoxicity of eugenol in isolated rat hepatocytes. *Chem.-Biol. Interact.* **1991**, *77*, 137−147.

(18) Jeng, J. H.; Hahn, L. J.; Lu, F. J.; Wang, Y. J.; Kuo, M. Y. P. Eugenol triggers different pathobiological effects on human oral mucosal fibroblasts. *J. Dent. Res.* **1994**, *73*, 1050−1055.

(19) Kozam, G.; Mantell, G. M. Effect of eugenol on oral mucous membranes. *J. Dent. Res.* **1978**, *57*, 954−957.

(20) Ghosh, R.; Nadiminty, N.; Fitzpatrick, J. E.; Alworth, W. L.; Slaga, T. J.; Kumar, A. P. Eugenol causes melanoma growth suppression through inhibition of E2F1 transcriptional activity. *J. Biol. Chem.* **2005**, *280*, 5812−5819.

(21) Han, E. H.; Hwang, Y. P.; Jeong, T. C.; Lee, S. S.; Shin, J. G.; Jeong, H. G. Eugenol inhibit 7,12-dimethylbenz[a]anthracene-induced genotoxicity in MCF-7 cells: Bifunctional effects on CYP1 and NAD(P)H:quinone oxidoreductase. *FEBS Lett.* **2007**, *581*, 749−756.

(22) Grespan, R.; Paludo, M.; Lemos Hde, P.; Barbosa, C. P.; Bersani-Amado, C. A.; Dalalio, M. M.; RK, C. Anti-arthritic Effect of Eugenol on Collagen-Induced Arthritis Experimental Model. *Biol. Pharm. Bull.* **2012**, *35*, 1818−1820.

(23) Kamatou, G. P.; Vermaak, I.; Viljoen, A. M. Eugenol-from the remote maluku Islands to the international market place: a review of a remarkable and versatile molecule. *Molecules* **2012**, *17*, 6953−6981.

(24) Woolverton, C. J.; Fotos, P. G.; Mokas, M. J.; ME, M. Evaluation of eugenol for mutagenicity by the mouse micronucleus test. *J. Oral Pathol. Med.* **1986**, *15*, 450−453.

(25) Sukumaran, K.; R, K. Inhibition of tobacco-induced mutagenesis by eugenol and plant-extracts. *Mutat. Res., Genet. Toxicol.* **1995**, *343*, 25−30.
(26) Huang, P.; Li, K.; Xu, Y.-Q.; Wang, Z.-J.; Liu, S.-S. Genetically modified *Caenorhabditis elegans* may lead to inaccurate toxicity evaluation of mixtures. *Environ. Sci. Eur.* **2020**, *32*, 63.
(27) Feng, L.; Liu, S.-S.; Li, K.; Tang, H.-X.; Liu, H.-L. The time-dependent synergism of the six-component mixtures of substituted phenols, pesticides and ionic liquids to *Caenorhabditis elegans*. *J. Hazard. Mater.* **2017**, *327*, 11−17.
(28) Atsumi, T.; Fujisawa, S.; Tonosaki, K. A comparative study of the antioxidant/prooxidant activities of eugenol and isoeugenol with various concentrations and oxidation conditions. *Toxicol. In Vitro* **2005**, *19*, 1025−1033.
(29) Li, J.; Yu, Y.; Yang, Y.; Liu, X.; Zhang, J.; Li, B.; Zhou, X.; Niu, J.; Wei, X.; Liu, Z. A 15-day oral dose toxicity study of aspirin eugenol ester in Wistar rats. *Food Chem. Toxicol.* **2012**, *50*, 1980−1985.
(30) Tang, H.-X.; Liu, S.-S.; Li, K.; Feng, L. Combining the uniform design-based ray procedure with combination index to investigate synergistic lethal toxicities of ternary mixtures on *Caenorhabditis elegans*. *Anal. Methods* **2016**, *8*, 4466−4472.
(31) Moyson, S.; Vissenberg, K.; Fransen, E.; Blust, R.; Husson, S. J. Mixture effects of copper, cadmium, and zinc on mortality and behavior of *Caenorhabditis elegans*. *Environ. Toxicol. Chem.* **2018**, *37*, 145−159.
(32) Verdú, S.; Ruiz-Rico, M.; Perez, A. J.; Barat, J. M.; Talens, P.; Grau, R. Toxicological implications of amplifying the antibacterial activity of gallic acid by immobilisation on silica particles: A study on C. elegans. *Environ. Toxicol. Pharmacol.* **2020**, *80*, No. 103492.
(33) De la Parra-Guerra, A.; Sturzenbaum, S.; Olivero-Verbel, J. Intergenerational toxicity of nonylphenol ethoxylate (NP-9) in *Caenorhabditis elegans*. *Ecotoxicol. Environ. Saf.* **2020**, *197*, No. 110588.
(34) Qiu, J.; Feng, H.; Lu, J.; Xiang, H.; Wang, D.; Dong, J.; Wang, J.; Wang, X.; Liu, J.; Deng, X. Eugenol reduces the expression of virulence-related exoproteins in *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **2010**, *76*, 5846−5851.
(35) Zhang, J.; Ding, T.-T.; Dong, X.-Q.; Bian, Z.-Q. Time-dependent and Pb-dependent antagonism and synergism towards *Vibrio qinghaiensis* sp.-Q67 within heavy metal mixtures. *RSC Adv.* **2018**, *8*, 26089−26098.
(36) Zhang, J.; Liu, S.-S.; Xiao, Q.-F.; Huang, X.-H.; Chen, Q. Identifying the component responsible for antagonism within ionic liquid mixtures using the up-to-down procedure integrated with a uniform design ray method. *Ecotoxicol. Environ. Saf.* **2014**, *107*, 16−21.
(37) Fan, Y.; Liu, S.-S.; Qu, R.; Li, K.; Liu, H.-L. Polymyxin B sulfate inducing time-dependent antagonism of the mixtures of pesticide, ionic liquids, and antibiotics to *Vibrio qinghaiensis* sp.-Q67. *RSC Adv.* **2017**, *7*, 6080−6088.
(38) Kumar, A.; Siddiqi, N. J.; Alrashood, S. T.; Khan, H. A.; Dubey, A.; Sharma, B. Protective effect of eugenol on hepatic inflammation and oxidative stress induced by cadmium in male rats. *Biomed. Pharmacother.* **2021**, *139*, No. 111588.
(39) Pinheiro, F. G.; Moreira-Gomes, M. D.; Machado, M. N.; Almeida, T. D. S.; Barboza, P.; Silva Oliveira, L. F.; Avila Cavalcante, F. S.; Leal-Cardoso, J. H.; Fortunato, R. S.; Zin, W. A. Eugenol mitigated acute lung but not spermatic toxicity of C60 fullerene emulsion in mice. *Environ. Pollut.* **2021**, *269*, No. 116188.
(40) How, S. H.; Ng, T. H.; Kuan, Y. C.; Jamalludin, A. R.; Fauzi, A. R. Survival of lung cancer patients in a resource-limited country. *Asia-Pac. J. Clin. Oncol.* **2015**, *11*, 221−227.
(41) Mohammadi Nejad, S.; Ozgunes, H.; Basaran, N. Pharmacological and toxicological properties of eugenol. *Turk. J. Pharm. Sci.* **2017**, *14*, 201−206.
(42) Hemaiswarya, S.; Doble, M. Synergistic interaction of eugenol with antibiotics against Gram negative bacteria. *Phytomedicine* **2009**, *16*, 997−1005.
(43) Girard, L. R.; Fiedler, T. J.; Harris, T. W.; Carvalho, F.; Antoshechkin, I.; Han, M.; Sternberg, P. W.; Stein, L. D.; Chalfie, M. WormBook: the online review of *Caenorhabditis elegans* biology. *Nucleic Acids Res.* **2007**, *35*, D472−D475.
(44) Wang, Y.; Liu, S.-S.; Huang, P.; Wang, Z.-J.; Xu, Y.-Q. Assessing the combined toxicity of carbamate mixtures as well as organophosphorus mixtures to *Caenorhabditis elegans* using the locomotion behaviors as endpoints. *Sci. Total Environ.* **2021**, *760*, No. 143378.
(45) Liu, S.-S.; Zhang, J.; Zhang, Y.-H.; Qin, L.-T. APTox: Assessment and prediction on toxicity of chemical mixtures. *Acta Chim. Sin.* **2012**, *70*, 1511−1517.
(46) Li, T.; Liu, S.-S.; Qu, R.; Liu, H.-L. Global concentration additivity and prediction of mixture toxicities, taking nitrobenzene derivatives as an example. *Ecotoxicol. Environ. Saf.* **2017**, *144*, 475−481.
(47) Liu, S.-S.; Xiao, Q.-F.; Zhang, J.; Yu, M. Uniform design ray in the assessment of combined toxicities of multi-component mixtures. *Sci. Bull.* **2016**, *61*, 52−58.
(48) Huang, P.; Liu, S.-S.; Xu, Y.-Q.; Wang, Y.; Wang, Z.-J. Combined lethal toxicities of pesticides with similar structures to *Caenorhabditis elegans* are not necessarily concentration additives. *Environ. Pollut.* **2021**, *286*, No. 117207.
(49) Tao, M.-T.; Bian, Z.-Q.; Zhang, J.; Wang, T.; Shen, H.-Y. Quantitative evaluation and the toxicity mechanism of synergism within three organophosphorus pesticide mixtures to *Chlorella pyrenoidosa*. *Environ. Sci.: Processes Impacts* **2020**, *22*, 2095−2103.

https://doi.org/10.1021/acsomega.2c03577
ACS Omega 2022, 7, 32238−32249