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Evaluation of food-relevant chemicals in the ToxCast high-throughput screening program

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ABSTRACT

Thousands of chemicals are directly added to or come in contact with food, many of which have undergone little to no toxicological evaluation. The landscape of the food-relevant chemical universe was evaluated using cheminformatics, and subsequently the bioactivity of food-relevant chemicals across the publicly available ToxCast highthroughput screening program was assessed. In total, 8659 food-relevant chemicals were compiled including direct food additives, food contact substances, and pesticides. Of these food-relevant chemicals, 4719 had curated structure definition files amenable to defining chemical fingerprints, which were used to cluster chemicals using a selforganizing map approach. Pesticides, and direct food additives clustered apart from one another with food contact substances generally in between, supporting that these categories not only reflect different uses but also distinct chemistries. Subsequently, 1530 food-relevant chemicals were identified in ToxCast comprising 616 direct food additives, 371 food contact substances, and 543 pesticides. Bioactivity across ToxCast was filtered for cytotoxicity to identify selective chemical effects. Initiating analyses from strictly chemical-based methodology or bioactivity/cytotoxicity-driven evaluation presents unbiased approaches for prioritizing chemicals. Although bioactivity *in vitro* is not necessarily predictive of adverse effects *in vivo*, these data provide insight into chemical properties and cellular targets through which foodrelevant chemicals elicit bioactivity.

1. Introduction

An estimated ~10,000 chemicals are directly or indirectly added to food in the United States, serving to enhance and preserve the taste and appearance of foods, prevent spoilage, or act as packaging constituents (Neltner et al., 2013). The addition of such chemicals to human food is allowed by the US Food and Drug Administration (FDA) under the 1958 US Food Additives Amendment. The safe use of new chemicals added to food is determined based on "reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use" (21 CFR §170.30) (Rulis and Levitt, 2009). However, for additives commonly used before 1958, safety may have been based on past use/experience rather than scientific data (12 USC §321) (Burdock and Carabin, 2004). Thus, with thousands of food-relevant chemicals approved for use in food, and as many as ~70% of direct additives having minimal to no toxicological guideline study data (Neltner et al., 2013), approaches that offer rapid evaluation to help inform, maintain, and support food safety are needed.

In vitro high-throughput screening (HTS) assays offer a time- and cost-effective platform for the evaluation of large chemical libraries (National Research Council, 2007). Such assays, in combination with computational approaches, provide an opportunity to rapidly gain insight into chemical-elicited effects on biochemical endpoints, cellular processes, and phenotypes as well as support

Abbreviations: CASRN, chemical abstract services registration number; EAFUS, Everything Added to Food in the US; EPA, US Environmental Protection Agency; ER, estrogen receptor; FDA, US Food and Drug Administration; FEMA, Flavor & Extract Manufacturers Association; GRAS, generally recognized as safe; HTS, high-throughput screening; MIE, molecular initiating event; NDGA, nordihydroguaiaretic acid; QSAR, quantitative structure–activity relationship; SDF, structure definition file; SMILES, simplified molecular input line entry system code; SOM, self-organizing map.

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the 3 R's (replacement, reduction, and refinement) of animal use in toxicological testing (Russell and Burch, 1958; Ankley et al., 2010b; Thomas et al., 2013). Using HTS data to inform on bioactivity can have great potential impact on both product development and safety testing (i.e., provide a platform for cost-effective high-throughput hazard identification). A significant example is the US Environmental Protection Agency's (EPA) ongoing ToxCast HTS program, which has evaluated a library of over 3000 chemicals in concentration-response format across over 1000 targeted *in vitro* assay endpoints to assess bioactivity *in vitro* (Dix et al., 2007; Kavlock et al., 2012; EPA, 2016b). A subset of 800 chemicals in ToxCast, termed the E1K library, were specifically screened across a subset of endocrine-related endpoints, highlighting that not all chemicals were evaluated in all ToxCast assays. The ToxCast assays cover a broad spectrum of chemical effects including enzyme inhibition, interaction with receptors, induction of cell stress pathways, and overt cytotoxicity (Kavlock et al., 2012).

The study presented herein is the first to evaluate strictly food-relevant chemicals across the entire ToxCast HTS program. Initially, a comprehensive inventory of food-relevant chemicals was compiled identifying 8659 food-relevant chemicals that was divided into three lists based not only on use but also chemistry: (1) direct food additives, (2) food contact substances, and (3) pesticides. The compiled food-relevant chemical list was then mined against the entire ToxCast chemical inventory identifying 1530 food-relevant chemicals evaluated in ToxCast. The bioactivity of these 1530 chemicals across all tested assay endpoints was assessed, and we demonstrated that filtering bioactivity using cytotoxicity can help hone in on potential selective chemical-mediated bioactivity to aid in prioritization and characterization of chemical effects. Combined, the results suggest that large HTS programs such as ToxCast are a valuable resource that can help inform on chemical prioritization and can have potential use as support for food safety testing.

2. Materials and methods

2.1. Identification of food-relevant chemicals

The inventory from publicly accessible databases was mined for chemicals, identified by their chemical abstract services registration numbers (CASRNs), to compile a comprehensive list of chemicals having any use associated with food. Accessed databases included the following FDA resources: Everything Added to Food in the US (EAFUS) (FDA, 2016a); Generally Recognized as Safe (GRAS) Notice Inventory (FDA, 2016a); Select Committee on GRAS Substance Database (SCOGS) (FDA, 2016c); List of Indirect Additives Used in Food Contact Substances (FDA, 2015); Inventory of Effective Food Contact Substances (FDA, 2016b); and Threshold of Regulation (TOR) Exemptions (FDA, 2016d). In addition, the Flavor & Extract Manufacturers Association GRAS inventory (FEMA, 2016) and the Aland Wood Pesticide database comprising active ingredients in pesticides which were assumed to be food use for the purpose of this study (Wood, 2015) were also included. Any defined chemical mixtures encountered were separated into the individual components and listed as unique CASRN for the purposes of this study. The compiled list of food-relevant chemicals including all source lists are summarized in Supplementary File S1, and a summary of the inventories is provided in Table 1. The food-relevant chemical list was cross-referenced against the entire publicly available ToxCast program chemical inventory comprising 3784 chemicals (EPA, 2016b). More specifically, the "ToxCast & Tox21 Chemicals Distributed Structure Searchable Toxicity Database (DSSTox files)" dataset (DSSTox_20151019 released October 2015) was downloaded; chemicals evaluated in ToxCast were obtained from "DSSTox_ToxCastRelease_20151019.xlsx".

2.2. Chemical clustering

Manually curated, high-quality, quantitative structure–activity relationship (QSAR)–ready simplified molecular input line entry system codes (SMILES) curated by Mansouri et al. were obtained from DSSTox for 4719 of the 8659 food-relevant chemicals (EPA, 2015; Mansouri et al., 2016). More specifically, the DSSTox Data was downloaded and SMILES were retrieved from the "DSSTox-All_20151019.xlsx" file. DSSTox does not contain SMILES for metals, polymers, and unstable stereoisomers as they were not amenable to the requirements for QSAR-ready structure definition file (SDF) generation, and were omitted from these analyses. Furthermore, it is important to note that while SMILES may exist for more of the food-relevant chemicals, the current study only obtained SMILES from DSSTox for consistency and reliability as DSSTox is a trustworthy manually curated resource. Using the rcdk package in R software (Guha, 2007), the SMILES were used to generate SDFs from which fingerprints were subsequently calculated using the same rcdk package. The generated molecular fingerprints describe a chemical's structure in a series of zeros or ones representing the presence or absence of a substructure descriptor which were defined using two descriptor sets: MACCS comprised of 166 descriptors and PubChem comprised of 881 descriptors. The MACCS descriptors are commonly used to evaluate chemical similarity describing general chemical substructure features, the PubChem fingerprints also describe substructural features summarizing a diversity of structural valence-bond forms. In total, 874 descriptors were associated with at least one food-relevant chemical and hence included for analysis (162 from MACCS and 712 from PubChem). The kohonen package in R (Wehrens and Buydens, 2007) was used to cluster the chemicals based on fingerprints across the 874 descriptors provided to the algorithm to form a self-organizing map (SOM), which groups the most similar chemicals together and displays cluster relationships in map form. The SOM generated from the 4719 chemicals was used to visualize chemical use categories as well as the ToxCast results. Supplementary File S2 provides the CASRN and SMILES for the 4719 chemicals in the SOM as well as which bin each chemical was in after clustering. Supplementary File S3 provides performance metrics from the SOM clustering. All analyses were conducted in R v3.1.3, with all scripts including analysis and each figure's code compiled into the source package "karmaus.fct.2016" attached as Supplementary File S4.

2.3. ToxCast HTS data

ToxCast data were retrieved from the publicly available download files (EPA, 2016b). For reproducibility, a self-contained R package with all pertinent data, analysis scripts, and figure generation scripts is provided as Supplementary File S4. Using the karmaus.fct.2016 R package, all data can be viewed, and all analyses and figures can be reproduced. To create this package, the "MySQL Database" (invitrodb_v2, released in October 2015) and the "R Package" (tcpl_1.0 released in November 2015) were downloaded and used as the foundation for all work (EPA, 2016b). Additionally, all the ToxCast data used for this study are also available for download as Excel files using the "ToxCast & Tox21 Summary Files" download link (for invitrodb_v2 released October 2015), the pertinent files used in the current study to evaluate ToxCast results are "tested_Matrix_151020.csv", "modl_ga_Matrix_151020.csv", "hitc_Matrix151020.csv", and "zscore_Matrix_151020.csv"; ToxCast data can also be viewed using the iCSS ToxCast Dashboard (EPA, 2016a). Chemicals evaluated in the ToxCast program were screened in concentration-response across 1157 assay endpoints,

Table 1
Summary inventories for compiled food-relevant chemicals and defined use categories.

Inventory source	Number of entries in inventory	Number of CASRN or codes in inventory[a]	Use category	Number of CASRN in use category[a,b]	Number of CASRN with defined fingerprints	Number of CASRN in ToxCast
FDA EAFUS	3968	3968 (3277)	**Direct Food Additives**	4610 → 4610 (3888 → 3888)	2016	616
FDA SCOGS	378	351 (320)				
FDA GRAS Notices	603	380 (349)				
FEMA GRAS	2796	2664 (2659)				
FDA Effective FCS	1205	715 (715)	**Food Contact Substances**	3785 → 3713 (3111 → 3039)	1173	371
FDA Indirect in FCS	3229	3229 (2555)				
FDA TOR	50	56 (56)				
Alan Wood Pesticides	1813	1808 (1808)	**Pesticides**	1808 → 1732 (1808 → 1732)	1530	543
TOTAL	14,042	13,171 (11,733)	**TOTAL**	13,171 → 10,055 (11,733 → 8659)	4719	1530

[a] The values preceding brackets represent the sum of unique CASRN and FDA CFSAN-generated numeric codes (ie. 977nnnnnn) together. However, since the FDA codes do not reflect true chemical entities mapped to CASRN, the numbers in brackets omit these codes and summarize the total number of unique CASRN only.
[b] The Total Number CASRN in use category reflects the sum of CASRN in each category. The values before the arrow represent the number of CASRN in each use category when merging the inventories from the multiple sources contributing to the use category. The values after the arrow represent the unique number of CASRN in each use category after chemicals were assigned to only one use category, and all duplicates were removed. For chemicals appearing in more than one use category preference was given to classification as a Direct Food Additive > Food Contact Substance > Pesticide. The subsequent columns ("Number of CASRN with Defined Fingerprints" and the "Number of CASRN in ToxCast" are subsets from the final value after the arrow in brackets.

though not all chemicals were evaluated in all assay endpoints. For example, most of the 1530 food-relevant chemicals were evaluated in ~300–800 assay endpoints, with a range of assay endpoints tested per food-relevant chemical from as many as 1057 assay endpoints to as few as 95 assay endpoints. Chemicals with fewer tested assay endpoints generally comprise the E1K subset of chemicals that were prioritized for the EPA's Endocrine Disruptor Screening Program and run mainly in endocrine-related assays (Browne et al., 2015). The significant effect of a chemical in any assay (referred to as the bioactivity of a chemical for the purposes of this study) was already identified in the downloaded data, denoted by the "hitc" output variable from analysis with the ToxCast Pipeline package in R (tcpl package, version 1.0) (EPA, 2016b) such that hitc was true when a concentration–response relationship and a minimum activity threshold were achieved. For the purposes of this study, the AC_{50} value (identified as the "modl_ga" variable from the downloaded data) was used as a quantitative measure to reflect the potency of bioactivity. The AC_{50} value reflects the concentration at which 50% of the maximum response is achieved (where response units could be fold change, percent activity, or percent viability).

2.4. Cytotoxicity evaluation

Because cytotoxicity can be a confounding factor in the evaluation of specific *in vitro* assay effects (Cantor and Janovitz, 2013), we defined the concentration at which chemicals elicited overt cytotoxicity. The median AC_{50} from 35 ToxCast assays measuring cytotoxicity was used to define a "cytotoxicity center" for each chemical (Supplementary File S5 summarizes the 35 cytotoxicity assays and cytotoxicity centers per chemical). All 1530 chemicals were evaluated in at least 14 of the 35 cytotoxicity assays (i.e., not all chemicals were evaluated in all cytotoxicity assays). To calculate a cytotoxicity center, chemicals must have elicited a significant effect in at least three cytotoxicity assays. For the purpose of filtering out bioactivity that may have been confounded by cytotoxicity, we defined a "cytotoxicity limit" by multiplying the global median absolute deviation (MAD) of all chemicals across all assay endpoints by 3. This approach sets the cytotoxicity limit based on the distribution of variances between AC_{50} values across the cytotoxicity assays. By applying the cytotoxicity limit as a threshold, we defined selective bioactivity as any assay endpoint with an AC_{50} below the cytotoxicity limit. This approach recognizes that all bioactivity occurs within a distribution and aims to conservatively define selective bioactivity for each chemical.

3. Results

3.1. Chemical clustering

A list of 8659 chemicals was identified as food-relevant comprising a diversity of uses and chemistries. To organize this large list, three use categories were defined per chemical based on the database of origin: (1) direct additives, (2) food contact substances, and (3) pesticides (Table 1). Several inventories contained code numbers generated by FDA in lieu of CASRN to identify samples that are not attributable to specific CASRN; such codes were removed from the total chemical counts as these entries do not reflect defined chemical entities. Furthermore, for the purposes of this study, chemicals were only allowed in one use category with priority given to direct food additives > food contact substances > pesticides. These requirements resulted in a workflow beginning with 13,171 unique total CASRN or codes compiled from all resources, reduced to 10,055 CASRN, and ultimately once chemicals were only allowed into one use category 8659 unique food-relevant chemicals. More specifically, the direct food additives category contains 3888 substances that were obtained from the FDA SCOGS, FDA GRAS, FEMA GRAS, and EAFUS databases; these chemicals are generally added to food to achieve an intended technical effect in the finished food. The food contact substance category comprises 3039 chemicals from food contact substance databases that are used in packaging, manufacturing, and transportation of food, but have no intended technical effect in or on finished food, nor are they necessarily intended to be in the finished food product. Finally, the pesticides category comprised the entire Alan Wood database of 1808 pesticide actives, of which 76 chemicals were duplicated from other sources and assigned into other use categories, resulting in 1732 chemicals being classified as pesticides for the purpose of the current study. There were 3080 chemicals that were identified in multiple databases sources, this overlap is largely represented by the 2348 chemicals that overlap between FEMA GRAS and EAFUS.

To evaluate the landscape of structural diversity among the food-relevant chemicals, fingerprints were generated for 4719 of the 8659 food-relevant chemicals which had SMILES available in

DSSTox, and used to build a SOM. The SOM clustering approach organized chemicals into bins, effectively grouping the large list of chemicals based on fingerprints (ie. physical/chemical similarity). A layout of 20 × 24 was used to target ~10 chemicals per bin (i.e., 480 bins for 4719 chemicals). The resulting clustering achieved a stable clustering distribution with an average of 10 chemicals per bin, with a distribution ranging from 0 to 50 chemicals per bin, Supplementary File S3 contains more details on the summary statistics for the SOM. For visualization, bins were laid out such that those containing most alike chemicals were nearest to each other, with direct neighboring bins being most similar. This SOM was then colored to visualize the use category designations of the chemicals in each bin— food additives, food contact substances, or pesticides. The proportion of chemicals in each bin belonging to the respective use categories revealed a clear separation such that distinct regions of the SOM comprised 100% direct additives or pesticides while the food contact substances were distributed between these regions (Fig. 1A–C). It is important to note that the percent of chemicals per use category may be affected by the total number of chemicals in each bin. However, the layout of bins will be driven by similarity regardless of the number of chemicals in each bin, reflecting the key descriptors/fingerprints that define the bins. The clear separation of direct additives from pesticides, with food contact substances spread in between, supported the designation of use categories as a functional grouping for chemicals by confirming that there are distinct physical/chemical properties differentiating these groups of chemicals.

The same SOM was also used to visualize chemicals evaluated in ToxCast. First, the number of chemicals per bin evaluated in ToxCast was highlighted (Fig. 1D). There were 1475 of the 1530 food-

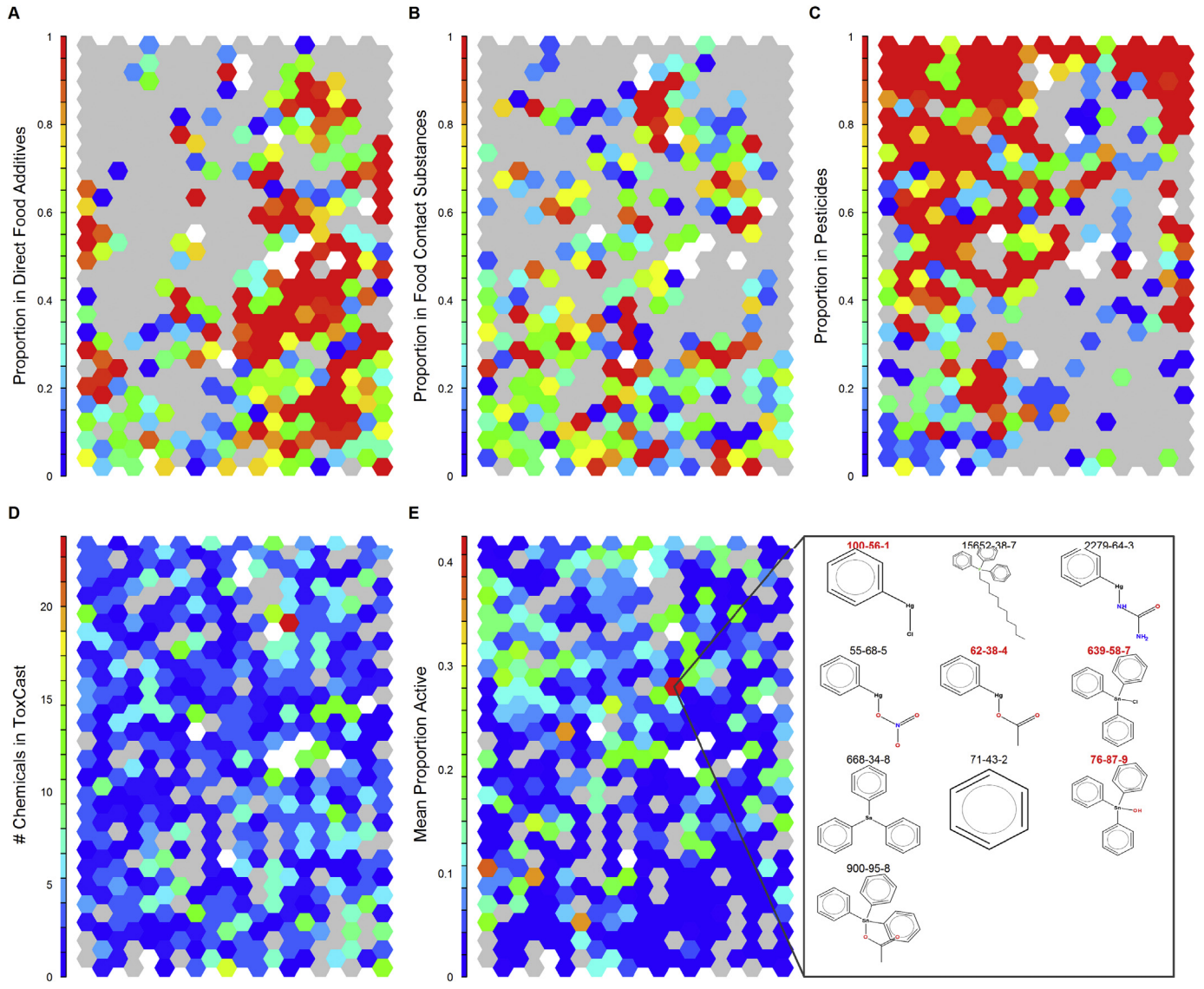

Fig. 1. SOM colored to evaluate chemical use and bioactivity. The SOM generated using 4719 food-relevant chemicals was highlighted to visualize the proportion of chemicals per bin that are (A) direct food additives, (B) food contact substances, or (C) pesticides. The color scale ranges from blue to red such that bins with no chemicals associated with the use category are gray and bins where all chemicals are in the use category are red. There are 12 empty bins with no chemicals (white). (D) To visualize ToxCast coverage and bioactivity, bins were then highlighted to reflect the number of chemicals in ToxCast, such that gray bins are composed entirely of chemicals not evaluated in ToxCast. (E) Finally, the relative bioactivity in ToxCast shown as the mean of the proportion bioactive assays was highlighted for tested chemicals (i.e., mean of the number of active assays out of total number of assays in which the chemical was tested). The bin with the greatest mean proportion of active assays is expanded, revealing that 4/10 chemicals in this bin were evaluated in ToxCast (bold red CASRN) with a mean of 0.4 proportion active assays, suggesting that these chemicals are highly promiscuous in *in vitro* assays. CASRN, chemical abstract services registration number; SOM, self-organizing map.

relevant chemicals in ToxCast that had structural data in DSSTox amenable to clustering for the SOM. Although not all bins contain a chemical represented in ToxCast, most of the food-relevant chemical landscape represented in the SOM is included in ToxCast with 395 of the 468 bins containing chemicals (480 bins total minus 12 empty bins) having at least one chemical evaluated in ToxCast. This demonstrates that the ToxCast chemical inventory encompasses a broad diversity of food-relevant chemistry without a discernable void in coverage. Finally, to evaluate bioactivity, the mean proportion of active assay endpoints per chemical (calculated as the number of active assay endpoints divided by the total number of assay endpoints in which the chemical was tested) was visualized using heatmap coloring on the SOM (Fig. 1E). Overall, the bins in the bottom-right corner, composed largely of pesticides, had the highest proportion of active chemicals. Some bins stood out as having a larger proportion of assay endpoints with significant bioactivity, suggesting that fingerprints comprising these bins may be associated with chemicals eliciting promiscuous bioactivity. The bin with the highest proportion of active assay endpoints was expanded revealing triphenyltins and phenylmercuric chemicals all belonging to the pesticides category (Fig. 1E). It is important to note that there are 10 chemicals comprising this bin with only four having been evaluated in ToxCast (designated by the CASRN in bold red font).

Having evaluated chemical similarity and proportion of active assay endpoints, a ternary plot was used to visualize mean potency of bioactivity in ToxCast to gain insight into the relative potency of chemicals across use categories (Fig. 2). This approach also utilizes the same bins that chemicals were grouped into from the SOM, using only the 395 bins that contained at least one chemical evaluated in ToxCast. These bins were plotted as points across three axes representing the different use categories to integrate separation based on the proportion of chemicals in each bin belonging to each category (integrating the results depicted in Fig. 1A–C). The points were then colored to visualize mean sum potency, calculated as the sum of $-\log(AC_{50})$, such that if a chemical was potently active in many assay endpoints it would have a greater sum. This plot reiterates that direct food additives and pesticides had the most distinct chemistries, as shown by the large number of bins directly on the top of those axes, respectively. Furthermore, the coloring reflecting potency reveals that bins composed of direct additives were mostly low potency chemicals versus the higher potency seen on the pesticides axis.

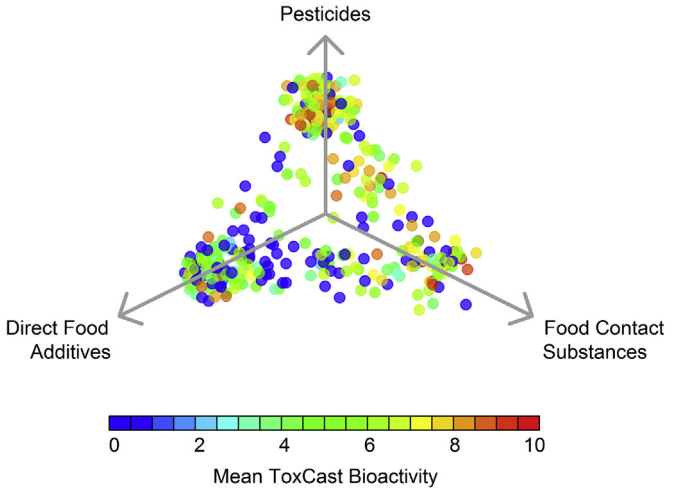

Fig. 2. Evaluation of mean activity in ToxCast for food-relevant chemicals. Chemicals were clustered into bins based on physical/structural fingerprints using a SOM algorithm. These bins were distributed on a ternary plot based on the proportion of chemicals in the bin belonging to each use category, such that the higher up, and closer to, the axis reflects greater proportion of chemicals in that bin belonging to the use category. To visualize overlapping points, translucent coloring and minimal scattering was incorporated. There are 395 bins represented on the plot, as at least one chemical had to be tested in ToxCast. Bins were colored based on the mean of the activity in ToxCast across the chemicals contained in the bin. Mean activity was calculated as the mean of log2 sum potency, where sum potency is the sum of $-\log(AC_{50})$ for all assays in which a chemical was bioactive. Direct food additives and pesticides had the largest number of bins with 100% of the chemicals belonging solely to those use categories, respectively, as visualized by the large cluster of bins at the top of those axes. The chemicals in the pesticide bins show greater mean bioactivity, revealed by more red and yellow bins; meanwhile the direct food additives are generally less bioactive, as shown by the density of blue and aqua bins on that axis.

3.2. Evaluation of cytotoxicity

Cytotoxicity and cell stress can confound results from cell-based *in vitro* assays. For example, in a loss-of-signal assay, a chemical that kills cells is very likely to concurrently decrease the assay signal, regardless of the relationship of the assay endpoint to the mechanism of cytotoxicity (Judson et al., 2013). To account for this effect, a cytotoxicity center was calculated for each chemical using the median AC_{50} from cytotoxicity assays in the ToxCast database. Although all 1530 food-relevant chemicals were evaluated in at least 14 cytotoxicity assays, not all chemicals elicited cytotoxicity in the concentration range tested (usually up to 100 μM). Only 25% of the food-relevant chemicals tested (388 of 1530) had a determined cytotoxicity center (Fig. 3). More specifically, only 10% (62 of 616) of direct food additives, 29% (108 of 371) of food contact substances, and 40% (218 of 543) of pesticides had determined cytotoxicity centers. These relative percentages demonstrate striking differences between the use categories. For chemicals where cytotoxicity centers were calculated, the cytotoxicity centers were generally between 10 and 100 μM with a slightly higher mean for direct food additives relative to the food contact substances and pesticides (Fig. 3). Several chemicals from each use category were outliers having significantly lower cytotoxicity centers; most of these outliers had cytotoxicity centers below 2 μM, suggesting that these chemicals may elicit more potent cytotoxicity and could be chemicals of interest (Table 2).

To eliminate possible promiscuous bioactivity concurrent with cell stress and cytotoxicity, bioactivity below a chemical-specific cytotoxicity limit was identified. In order to filter for bioactivity below the cytotoxicity limit, only chemicals that did elicit cytotoxicity (i.e., had a determined cytotoxicity center) were included. Application of the filter for cytotoxicity assumes that any bioactive assay endpoint with an AC_{50} value greater than or equal to the cytotoxicity limit was nonspecific and thus should no longer be included in the active assay count for the chemical. This filtering resulted in an 8-fold decrease in the average number of active assay endpoints per chemical for all use categories (from an average of ~80 assay endpoints per chemical to an average of ~10 assay endpoints per chemical). A noticeable trend showing pesticides having a higher mean number of active endpoints than food contact substances or direct additives can be seen (Fig. 4). Although the pesticides were on average evaluated in more assay endpoints than the chemicals in other use categories, the pesticides maintain a greater proportion of active assays and higher number of active assay endpoints overall, even when normalized to total number of assays tested (Supplementary File S6). In general, the chemicals with the greatest number of active assay endpoints in all use categories were those that elicited cytotoxicity, confirmed by the drastic decrease in active assay endpoints after cytotoxicity, which was consistent for chemicals across all use categories. The high number of active endpoints before filtering was similar across all use categories, ~80 assay endpoints; however, it is important to note that Fig. 4 is

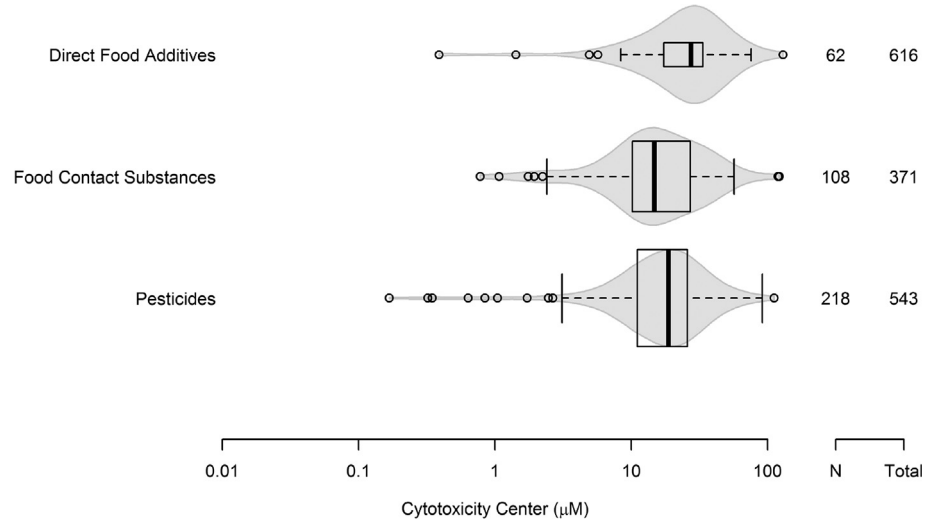

Fig. 3. Evaluation of cytotoxicity. The cytotoxicity range for food-relevant chemicals, grouped by use category, was determined based on 35 ToxCast cytotoxicity assays (note: all food-relevant chemicals were evaluated in at least 14 of these cytotoxicity assays). A minimum of three assays with concentration-dependent cytotoxicity effects were required per chemical, resulting in only a subset of chemicals having a determined cytotoxicity center. The number of chemicals with a determined cytotoxicity center (N) versus the total number of chemicals in each category are shown on the right. Boxplots represent the distribution of cytotoxicity center values, denoting the mean at the center of the boxplot and the 95th percentile within the boxplot whiskers. The relative height of the boxplots reflects the proportion of chemicals from the use category included in the plot (i.e., 10% [62 of 616] of direct additives had a determined cytotoxicity center resulting in a narrow boxplot versus 40% [218 of 543] of pesticides with a cytotoxicity center resulting in a tall box plot). The shaded area reflects the density of cytotoxicity AC_{50} values, to help visualize where the majority of data points lie.

Table 2
Chemicals with cytotoxicity centers below 2 μM.

CASRN	Chemical name	Active cytotoxicity assays/cytotoxicity assays evaluated	Cytotoxicity center (μM)[a]
Direct Food Additives			
8000-34-8	Clove leaf oil	4/35	0.39
112-31-2	Decanal	3/33	1.43
Food Contact Substances			
1461-22-9	Tributyltin chloride	34/35	0.78
683-18-1	Dibutyltin dichloride	15/19	1.07
81-48-1	D&C Violet 2	3/14	1.75
3064-70-8	Bis(trichloromethyl)sulfone	14/14	1.94
Pesticides			
50-65-7	Niclosamide	22/35	0.32
134-31-6	8-Hydroxyquinoline sulfate	4/19	0.35
76-87-9	Triphenyltin hydroxide	33/35	0.64
62-38-4	Phenylmercuric acetate	34/35	0.84
100-56-1	Phenylmercuric chloride	17/18	1.05
1897-45-6	Chlorothalonil	29/35	1.72

[a] The cytotoxicity center was calculated as the median of AC_{50} values from all active cytotoxicity assays in which the chemical was evaluated. Chemicals had to have a determined AC_{50} in $\geq$3 cytotoxicity assays for a cytotoxicity center to be calculated.

comprised only of chemicals for which cytotoxicity centers were determined so that cytotoxicity filtering would be possible, reflecting a far lower proportion of the direct food additives (10%) than food contact substances (29%) or pesticides (40%). The cytotoxicity filtered list of bioactive assay endpoints is more informative, providing better insight into potential selective targets of the chemical. For example, nordihydroguaiaretic acid (NDGA; CASRN 500-38-9) was active in 82 of the 317 assay endpoints in which it was tested with a determined cytotoxicity center of 52 μM based on AC50 values from 3 of the 19 cytotoxicity assays in which it was tested. Among the 82 active ToxCast assay endpoints, 30 had AC_{50} values below the cytotoxicity limit and were thus considered potential selective targets for NDGA. These bioactive assay endpoints after cytotoxicity filtering are the possible selective targets of NDGA and comprised 12 estrogen receptor (ER) assays, suggesting a potential mechanism of action as a xenoestrogen.

4. Discussion

Advances in analytical chemistry have paved the way for the detection and development of many chemicals and mixtures that are directly added to, and come in contact with, human food. These chemicals serve a multitude of purposes, including the fortification, preservation, manufacture, and packaging of food, and it has been suggested there are roughly 10,000 such chemicals in use in the United States (Neltner et al., 2013). We sought to comprehensively compile a list of chemicals from many publicly available resources to define a list of food-relevant chemicals. A total of 8659 unique food-relevant chemicals were identified, slightly below previous estimates of the food-use chemical universe; furthermore, only 3888 were possible direct additives while 4771 were food contact substances or pesticides. It is also important to note that all chemicals with GRAS registrations were systematically grouped into the direct food additives category for the purpose of our study; however, chemicals are given GRAS designation specifically based

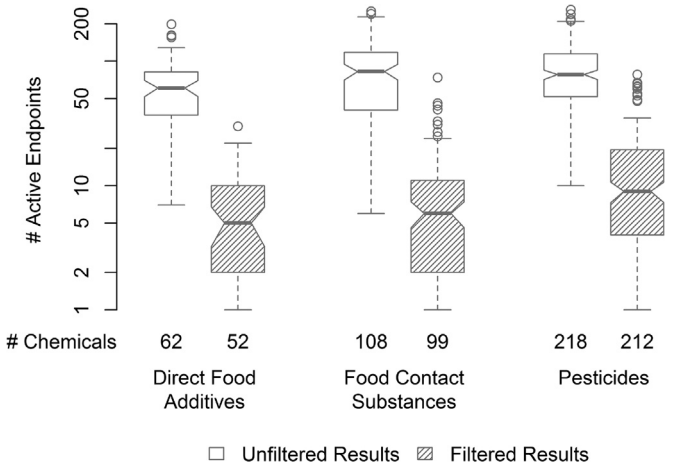

Fig. 4. Effect of cytotoxicity filtering. To identify the selective effects of chemicals from overt cytotoxicity (defined as having significant effects in ≥3 cytotoxicity assays), only bioactive assay endpoints with AC_{50} values below the cytotoxicity center were deemed active. This filtering approach resulted in a marked decrease in the number of active assays for these chemicals. On average, chemicals that elicited cytotoxicity were bioactive in an average of 80 assays per chemical (white; mean for direct food additives was 65 assay endpoints while both food contact substances and pesticides had a mean of 87 assay endpoints). However, when active assays having an AC_{50} above the cytotoxicity center per chemical were deemed nonspecific and removed from the bioactive assay count, the average number of selective bioactive assays was reduced 8-fold to an average of 10 assays per chemical (gray hashed; direct food additives average was 7 assay endpoints, food contact substances averaged 10 assay endpoints, and pesticides averaged 14 assay endpoints). There were a few chemicals that did not elicit any bioactivity below the cytotoxicity center, resulting in fewer chemicals included in the filtered versus unfiltered boxplots (see the number of chemicals listed below the boxplots).

on a defined intended use, which in the future should be evaluated for proper categorization and prioritization of these chemicals. Despite possible refinements in chemical categorization, our food-relevant list encompasses a broad chemical diversity including various uses representing unique chemistries as confirmed by SOM clustering. The list of 8659 food-relevant chemicals compiled herein is not necessarily complete, other resources could be included to increase the scope of food-use chemical analyses (e.g., incorporation of chemical lists from countries other than the United States).

With a defined list of 8659 food-relevant chemicals in hand, it becomes evident that traditional toxicity testing for each of these chemicals is simply not feasible. One suggested approach to address this challenge is to prioritize chemicals warranting follow-up testing using *in vitro* HTS (National Research Council, 2007). The cost- and time-efficient nature of HTS enables the evaluation of thousands of chemicals, even if there are limited to no previous testing data for a chemical. Publicly available data from the ToxCast *in vitro* HTS program as well as the Tox21 qHTS program offer unique resources for identifying the specific biochemical targets of chemicals, aiding in mapping adverse outcome pathways, and prioritizing chemicals for toxicological evaluation (Dix et al., 2007; EPA, 2016b; NIH/NCATS, 2016). The Tox21 dataset, though rich in chemical diversity with ~9000 chemicals evaluated, was not included for the current study due to the lower number of assay endpoints evaluated. Our analysis is the first to specifically focus on food-relevant chemicals in ToxCast, identifying 1530 food-relevant chemicals. These 1530 chemicals showed trends in promiscuity and overt cytotoxicity between use categories, consistent with their design such that bioactivity across ToxCast correlated with the intended technical function of the ingredient. For instance, pesticide active ingredients are intended to have potent activity on an intended target by design, often growth inhibitory or toxic, whereas direct additives are not intended to have potent bioactivity.

The broad landscape of food-relevant chemical diversity and assay endpoints evaluated across ToxCast enables a multitude of analyses for the identification of specific chemicals of interest. Many suitable alternate and complementary approaches are possible for identifying chemicals of interest from such a large dataset in addition to the examples highlighted herein (clustering, cytotoxicity, and bioactivity summaries); nevertheless, our approaches revealed several points of interest. For example, NDGA was included in this study because it was listed in the EAFUS inventory due to its historic use as an antioxidant and preservative for fats and butter. Use of NDGA was initially approved by the Meat Inspection Division of the US War Food Administration in 1943 (Lu et al., 2010). The GRAS status for NDGA was withdrawn in 1968, because subsequent studies found that NDGA elicited nephropathy in rats (Evan and Gardner, 1979). This example highlights the fact that future studies would benefit from manual curation of large database outputs, as is the focus of our future studies. In the case of NDGA in the inventory, we discovered that several FDA databases serve as a repository for any/all registrations, whether historic and withdrawn or current and active. Interestingly, NDGA has more recently been shown to be estrogenic *in vitro* and *in vivo* and is being investigated for its potential medical use treating tamoxifen-resistant breast cancer due to its effect on the ER (Fujimoto et al., 2004; Zavodovskaya et al., 2008). These studies directly correlate with the top biological targets of NDGA in ToxCast, as after cytotoxicity filtering ER assays were identified as the most specific targets of NDGA.

Another example of potential priority chemicals identified in the current study are organotins, included in both the food contact substance and pesticide categories with uses including antifouling agents, stabilizers, and fungicides. Within the food-relevant subset of ToxCast, several organotins elicited potent cytotoxicity and bioactivity across assays in both the food contact substance and pesticide categories, namely tributyltin chloride (CASRN 1461-22-9) and triphenyltin hydroxide (CASRN 76-87-9), respectively. Furthermore, the SOM-based analysis identified the bin containing triphenyltins as having the highest proportion of active assays; this bin contained two organotins evaluated in ToxCast: triphenyltin chloride (CASRN 639-58-7) and triphenyltin hydroxide. Given that these chemicals, in addition to the phenylmercuric compounds in the same bin, had such a high proportion of active assays, it would be worthwhile to follow-up with evaluation of the other chemicals in this bin in future studies. It should be kept in mind, however, that these may represent a structural class that interfere with many *in vitro* assays (e.g., detergent-like compounds), and additional testing should be approached with this in mind (Judson et al., 2013). Evaluation of cytotoxicity center distributions identified the aforementioned organotins as outliers with significantly lower cytotoxicity centers, which artifactually increases the number of positive assay hits. Despite the triphenyltin compounds having been sorted into a different bin in the SOM than the tributyltin and dibutyltin compounds (likely due to the presence of the phenyl moiety), the merging of multiple analyses cumulatively identified all of these organotins. This finding from *in vitro* ToxCast data is consistent with the overt toxicity observed upon organotin exposure in animal studies (Kimbrough, 1976). In fact, the uses of organotins continue to be restricted, being limited to stabilizers for polyvinyl chloride packaging materials and fungicides for plant protection, resulting in very low potential food-use exposure. The highest risk of organotin consumption arises from eating shellfish, likely due to past use of such compounds as antifouling agents (Rosenberg, 2013).

In vitro assays can inform on chemical interaction with biochemical and cellular targets to aid in chemical hazard prioritization (Browne et al., 2015). However, it is important to note that *in vitro* assays also have limitations. Furthermore, *in vitro* assays are subject to false positives and negatives, such that the manual evaluation of data for endpoints of specific interest should always be considered. *In vitro* assays lack absorption, distribution, metabolism, and excretion processes that occur *in vivo* and may influence the bioavailability of a chemical in target tissues, which are critical to providing a dose context to the observed results. Significant advances have been made in modeling reverse toxicokinetics to aid in starting to address these challenges (Wambaugh et al., 2015; Wetmore et al., 2015), and added parameters should be considered with regard to indirect food contact substances to account for possible migration into products at levels that need to be defined. The resulting dietary exposures would need to be estimated in order to determine expected exposures. Such exposure estimates are a critical part of prioritization in addition to the characterization of hazard made possible by *in vitro* evaluation.

While the chemicals included in the current ToxCast inventory covered the food-relevant landscape fairly well despite comprising only chemicals soluble in dimethylsulfoxide (DMSO), no mixtures were evaluated in ToxCast. Food is composed of mixtures, though not all are well-defined mixtures. Most food contact substances were registered as defined mixtures, which could be emulated and evaluated in *in vitro* assays. Furthermore, assays in which food-relevant chemicals are most frequently active could be incorporated during the evaluation phase of ingredient development.

The battery of ~1000 assays included in the current ToxCast data is impressive, yet there are many biological targets that are not represented; new assays are being added to increase coverage of biological space (Kavlock et al., 2012). Ultimately, characterization of bioactivity through *in vitro* assays is merely one step in characterizing the effects of chemicals on biological systems. For instance, the construction of adverse outcome pathways (AOP) that link molecular initiating events (MIEs) measured with *in vitro* assays to apical endpoints is an area of increasing research and regulatory activity (Ankley et al., 2010a). The feasibility of the AOP approach has been demonstrated for the ER pathway in which a model based on *in vitro* assays targeting the MIE of ER activation was shown to robustly predict an associated *in vivo* adverse endpoint, increased uterine weight (Wambaugh et al., 2015). However, broader application of the AOP approach for endpoints that underlie most safety decisions for food-relevant chemicals is still in its infancy and will require additional effort across all sectors.

5. Conclusions

The identification of 8659 food-relevant chemicals and subsequent evaluation of 1530 diverse food-relevant chemicals across hundreds of *in vitro* assays demonstrated that large-scale analyses are possible and feasible for the food-relevant chemical universe. Data from the ToxCast HTS program provided the unique opportunity to evaluate the diversity of food-relevant chemicals in concentration-response on a broad set of endpoints. This is a significant step in characterizing the bioactivity of food-relevant chemicals *in vitro*. Evaluation of other alternate and/or complementary analysis methods and data sources would help build confidence in the ToxCast-based findings presented herein. Follow-up studies focusing on subsets of chemicals or subsets of targeted assays to address more specific topics of interest, incorporation of a cytotoxicity filter to identify specific chemical-mediated effects, and consideration of dosimetry and exposure estimates will also serve to strengthen the characterization of chemical-mediated effects identified *in vitro*. The current study highlighted examples of approaches for the large-scale identification and evaluation of food-relevant chemicals ultimately integrating cheminformatics and ToxCast HTS data. The results confirmed that such approaches are useful in identifying chemical-elicited bioactivity to support food safety when evaluated in the proper context. Future studies will seek to refine the categorization of food-relevant chemicals, incorporate other analysis and data integration approaches, and evaluate other available data sources such as exposure estimates and *in vivo* data to provide further context and confidence in the *in vitro* and *in silico* findings.

Disclaimer

The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the US Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Appendix A. Supplementary data

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Transparency document

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