Estrogenic Activity of Phenolic Additives Determined By an *In Vitro* Yeast Bioassay

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We used a recombinant yeast estrogen assay to assess the activity of 73 phenolic additives that are used as sunscreens, preservatives, disinfectants, antioxidants, flavorings, or for perfumery. Thirty-two of these compounds displayed activity: 22 with potencies relative to 17β-estradiol, ranging from 1/3,000 to < 1/3,000,000, and 10 compounds with an impaired response that could not be directly compared with 17β-estradiol. Forty-one compounds were inactive. The major criteria for activity appear to be the presence of an unhindered phenolic OH group in a *para* position and a molecular weight of 140–250 Da. *Key words*: estrogenic activity, phenolic additives, recombinant yeast assay. *Environ Health Perspect* 109:133–138 (2001). [Online 19 January 2001] *http://ehpnet1.niehs.nih.gov/docs/2001/109p133-138miller/abstract.html*

There is currently considerable interest worldwide in chemicals that are able to mimic estrogens (i.e., xenoestrogens). Because it is possible that exposure to xenoestrogens may (and only may) lead to adverse physiologic effects in humans and wildlife, intense efforts are under way to identify chemicals that possess estrogenic activity. To date, no large-scale, systematic screening of chemicals for estrogenic activity has been conducted (although one is planned) (*1*). Instead, the estrogenic activity of some chemicals has been discovered by accident, usually because estrogenic effects have been observed in groups of animals exposed to high doses or concentrations of chemicals (*2*), or by small-scale screening, for example, alkylphenols (*3,4*) and phthalates (*5,6*).

The identification of many structurally diverse chemicals possessing (usually weak) estrogenic activity has allowed structure–activity relationships (SARs) to be developed [e.g., Waller et al. (*7*)]. To date, these models have not been used in a predictive sense (i.e., to identify chemicals likely to have estrogenic activity), but they have been useful in identifying the major structural features associated with estrogenic activity. Because receptor binding is based on the hypothesis that all active molecules interact with the receptor site in the same or similar mode, a similar pattern of atoms or functional groups in the compounds activating receptor sites is usually required to facilitate recognition and binding. In the case of the human estrogen receptor site, a ligand with a phenolic group seems to be a common feature of most, but not all, molecules that display a substantial binding activity (*8,9*). On the basis of this knowledge, we screened a selection of commercially used phenolic additives to assess their estrogenic activity. Phenolic additives are used without modification in widely different end products, and in this respect, they differ from most phenols that are used in the synthesis of other organic chemicals, frequently as components of polymeric materials.

Materials and Methods

Selection of chemicals. We characterized the phenolic additives by searching directories of commercial chemicals displaying chemical structures (*10–13*). The preliminary search generated a selection of several hundred compounds, but we reduced this number by excluding drugs, dyes, and those phenols used as precursor chemicals for manufacturing other compounds. There were about 140 compounds remaining; we reduced this group to 73 by investigating only compounds that were available in a high-purity form from suppliers. We confirmed the purity of the compounds by HPLC; in the few cases where impurities apparently exceeded 2%, compounds were recrystallized. We believe that the compounds finally tested are a representative, but not comprehensive, selection of phenols that are incorporated without modification into a wide range of commercially important products. They fell into the following classes: UV screening agents, preservatives, disinfectants, antioxidants, and flavoring and perfumery components. The chemicals tested are listed in Tables 1–5.

Assessment of estrogenic activity. Details of the yeast estrogenicity assay (including details of the medium components) have been previously described (*14*). In brief, yeast cells transfected with the human estrogen receptor α (ERα) gene, together with expression plasmids (containing estrogen responsive elements and the *lac*-Z reporter gene encoding the enzyme β-galactosidase), were incubated in medium containing the test chemical and the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG). Active ligands (which bind to the receptor) induce β-galactosidase (β-gal) expression, and this causes the CPRG (initially yellow) to change into a red product that can be measured by absorbance.

Stock solutions of chemicals (dissolved in ethanol) were serially diluted in ethanol, and 10 µL volumes were transferred to 96-well, flat-bottom plates. After the ethanol was allowed to evaporate to dryness, 200 µL medium containing CPRG and yeast was added to each well. The plates were then incubated at 32°C for 3 days, after which absorbance readings were made at 540 nm using a Spectramax 340 PC plate reader (Molecular Devices, Sunnyvale, CA). We included 17β-estradiol (serially diluted from 1×10^{-8} M to 4.88×10^{-12} M) and solvent controls in each assay. Each chemical was tested at least twice. The median effective dose (ED_{50}) for 17β-estradiol was 2.0×10^{-10} M ± 0.22×10^{-10} M (mean ± SE of 14 experiments).

We determined relative potencies of test chemicals only when the dose–response curves were parallel to that of 17β-estradiol. To do so, the concentration of the test chemical required to produce a half-maximal response (A_{540} between 1.7 and 2.0) was divided by the concentration of 17β-estradiol required to produce the same response. Compounds displaying a submaximal response were compared at the 10% response level.

Results

Tables 1–5 list the 73 phenolic additives tested in groups according to their use, together with a summary of the information generated by the *in vitro* estrogen assay. Thirty-two of the compounds tested displayed detectable estogenic activity. None was strongly active, and the most potent was 3,000 times less active than 17β-estradiol.

The response curves for the additives used as sunscreens or light stabilizers are shown in Figure 1 and are representative of the data obtained from all of the active chemicals. Twenty-two of the test compounds

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This work was supported in part under contract with the U.K. Department of Trade and Industry as part of the Government Chemist Programme.

Received 28 March 2000; accepted 11 September 2000.

produced full dose–response curves that were parallel to that of 17β-estradiol. Ten compounds generated submaximal response curves, a phenomenon that has previously been discussed (*15*). In some cases, submaximal response curves occured because the test chemical was toxic to the yeast at high concentrations (e.g., the dose–response curves for benzophenone-3 and benzophenone-7 in Figure 1A), but other chemicals generated shallower dose–response curves than 17β-estradiol for reasons not currently understood (e.g., menthyl salicylate, benzyl salicylate, and 2-ethylhexyl salicylate; Figure 1B). Such compounds were designated as having submaximal response in the tabulated results and are detailed in Table 6.

Discussion

The results summarized in Tables 1–5 show that activity has been detected in compounds falling into four out of the five usage categories, and only those used in flavoring and perfumery failed to display activity. The group of compounds used to screen UV light contained more estrogenically active compounds than the other groups, and so far, such compounds have not attracted much comment in this context. The structural diversity of the compounds investigated complicates the interpretation of the results, but a slightly clearer picture emerges if the active compounds are ranked in terms of their activity. Figures 2, 3, and 4, respectively, show the structures of phenolic additives with activities of 1/1,000–1/10,000, 1/10,000–1/100,000, and < 1/100,000.

The phenolic additives investigated in this study had from one to four nonfused aromatic rings in their structures; in some instances heterocyclic rings were also present. Those compounds found to be estrogenically active had only one or two nonfused aromatic rings in their structures, and with very few exceptions, one of these rings had a phenolic -OH group in a *para* position to an additional substituent. The structures of the active additives, shown in Figures 2, 3, and 4, make the importance of this configuration more obvious. The substituent may be an alkyl group; a chlorine atom; a methoxy group; or an ester, ketone, or C-C bond linking it to a second aromatic ring. The importance of the *para* position of the phenolic group is consistent with findings from a study of estrogenic activity of alkylphenolic compounds (*4*). The phenolic additives that were inactive in this study were chiefly those in which the phenolic -OH group was in an *ortho* position relative to other substituents, or where the 2,6 positions relative to the phenolic -OH group were occupied by other substituents, for example, additional -OH groups in the case of the gallates and purpurogallin; bromine atoms in the case of tetrabromobisphenol A; or *t*-butyl groups in the case of 4,4´-methylenebis(2,6,-di-*t*-butyl phenol) [Chemical Abstracts Service (CAS) no. 118-82-1], 2,6-di-*t*-butyl-4-(dimethylaminomethyl)phenol (CAS no. 88-27-7), octadecyl- 3-(3´,5´-di-*t*-butyl-4-hydroxyphenyl)propionate (CAS no. 2082-79-3), and 2,6 di-*t*-butylphenol. This suggests that where the hydrophilic phenol

Table 1. Phenolic additives used as sunscreens or light stabilizers.

Compound	CAS registry no.	Usage group	Estrogenic activity	MW
Benzophenone-1	131-56-6	1,2	1/3,000	214.2
Benzophenone-2	131-55-5	1,2	1/7,000	246.2
Benzophenone-3	131-57-7	1,2	Submax	228.3
Benzophenone-4	4065-45-6	1	ND	308.3
Benzophenone-6	131-54-4	2	Submax	274.3
Benzophenone-7	85-19-8	2	Submax	232.7
Benzophenone-8	131-53-3	1	ND	244.2
Benzophenone-12	1843-05-6	2	ND	326.5
4,4´-Dihydroxybenzophenone	611-94-4	2	1/40,000	214.2
Phenyl salicylate	118-55-8	1	1/300,000	214.2
Benzyl salicylate	118-58-1	1	Submax	228.3
Menthyl salicylate	89-46-3	1	Submax	276.4
Ethylhexyl salicylate	118-60-5	1	Submax	250.9
Triethanolamine salicylate	2174-16-5	1	ND	287.3
Resorcinol monobenzoate	136-36-7	2	1/80,000	214.2
Octrizole	3147-75-9	2	ND	323.4
2,4-Di-*t*-butyl-6(5-chloro-2H-benzotriazol-2-yl)phenol	3864-99-1	2	ND	357.9
7-Hydroxycoumarin	93-35-6	1	ND	162.1

Abbreviations: CAS, Chemical Abstracts Service; MW, molecular weight; ND, not detected; Submax, submaximal response curve; usage group 1, cosmetic sunscreen; usage group 2, light stabilizer for polymers. Estrogenic activity shows the potency relative to 17β-estradiol.

Table 2. Phenolic additives used as preservatives.

Compound	CAS registry no.	Estrogenic activity	MW
Dodecylparaben	2664-60-0	ND	306
Benzylparaben	94-18-8	1/4,000	228.2
Butylparaben	94-26-8	1/8,000	194.2
Propylparaben	94-13-3	1/30,000	180.2
Ethylparaben	120-47-8	1/200,000	166.2
Methylparaben	99-76-3	1/3,000,000	152.2
Dichlorophen	97-23-4	ND	269.1
2-Hydroxybiphenyl	90-43-7	1/2,000,000	170.2
4-Hydroxybiphenyl	92-69-3	1/10,000	170.2
Salicylic acid	69-72-7	ND	138.1

Abbreviations: CAS, Chemical Abstracts Service; MW, molecular weight; ND, not detected.

Table 3. Phenolic additives used as disinfectants.

Compound	CAS registry no.	Estrogenic activity	MW
Phenol	108-95-2	ND	94.1
2-Methylphenol	95-48-7	ND	108.1
4-Methylphenol	106-44-5	ND	108.1
Thymol	89-83-8	ND	150.2
Chlorothymol	89-68-9	1/400,000	184.7
4-*t*-Amylphenol	80-46-6	1/200,000	164.3
Carvacrol	499-75-2	ND	150.2
4-Chloro-3-methylphenol	59-50-7	1/3,000,000	142.6
4-Chloro-3,5-dimethylphenol	88-04-0	1/900,000	156.6
2,2'-Dihydroxybiphenyl	1806-29-7	ND	186.2
8-Hydroxyquinoline	148-24-3	ND	145.2

Abbreviations: CAS, Chemical Abstracts Service; MW, molecular weight; ND, not detected.

Table 4. Phenolic additives used in flavoring and perfumery.

Compound	CAS registry no.	Estrogenic activity	MW
Eugenol	97-53-0	ND	164.2
Isoeugenol	97-54-1	ND	164.2
Vanillin	121-33-5	ND	152.2
Ethyl vanillin	121-32-4	ND	166.2
Methyl salicylate	119-36-8	ND	152.2
Hexyl salicylate	6259-76-3	ND	222.3

Abbreviations: CAS, Chemical Abstracts Service; MW, molecular weight; ND, not detected.

group is hindered or deactivated, the receptor binding capability is greatly reduced.

17β-Estradiol has a molecular weight of 272.4 and its metabolites estrone and estriol have molecular weights of 270.4 and 288.4, with relative activities of one-half and 1/300 that of the parent compound, but Routledge and Sumpter (*4*) showed that the estrogenic activity of alkyl phenols peaked with 4-*t*-octylphenol, which has a molecular weight of 206.4. The series of phenolic additives examined in this study display much more structural variation than the alkyl phenols, but there does seem to be a similar optimum molecular weight. For example, the mean molecular weights of compounds falling within the three potency ranges of 1/1,000–1/10,000, 1/10,000–1/100,000, and < 1/100,000 are 208.2, 202.9 and 166.7, respectively. Figure 5, a scatter diagram in which molecular weight is plotted against the negative logarithm of the potency, shows a trend (correlation coefficient –0.74) in which activity diminishes as the molecular weight decreases from an optimum range of about 200–230. Clearly this trend is consistent with the concept of a receptor site that can accommodate molecules of the appropriate size, shape, and charge distribution, but molecular weight is a crude criterion because it does not take into account any aspect of the shape or charge distribution parameters. Nevertheless, when the inactive additives are considered, there were nine compounds that had an unhindered phenolic -OH group *para* to some other substituent; these were all found to have molecular weights of < 164 or > 302, which suggests that their lack of activity is probably size related (Figure 5). There is no apparent tendency for compounds with molecular weights exceeding about 250 to display a gradually diminishing activity. Instead there seems to be a sharp cutoff between active and inactive compounds, which is what might be expected if a size-exclusion mechanism excludes larger molecules from the estrogen receptor site. Unfortunately, in the group of compounds tested there were no compounds in that crucial molecular weight region with an unhindered phenolic -OH group *para* to some other substituent.

Thus, the two most important criteria to emerge from this study in relation to phenolic additives displaying estrogenic activity are *a*) having a phenol with a *para* configuration and *b*) having a molecule of appropriate size. Because the structural features of commercially important phenolic additives are so varied, it is only possible to cite a few illustrative examples from the data. For example, 4-hydroxybiphenyl is far more active than 2-hydroxybiphenyl; 4,4´-dihydroxybiphenyl is active and 2,2´-dihydroxybiphenyl is inactive; and insertion of a $-CH_2-$ or $-C(CH_3)_2-$group between the two aromatic rings of 4,4´-dihydroxybiphenyl [i.e., to give bis-(4-hydroxyphenyl) methane and bisphenol A] makes virtually no difference to the activity of the resulting compounds. Thymol, which has no substituent group in the *para* position to the phenolic -OH group, is inactive, whereas chlorothymol, which has a chloro group in that position, is active.

The benzophenones present a complex picture; this has been confirmed by Schultz et

Table 5. Phenolic additives used as antioxidants.

Compound	CAS registry no.	Estrogenic activity	MW
Gallic acid	149-91-7	ND	170.1
N-Propyl gallate	121-79-9	ND	212.2
N-Octyl gallate	1034-01-1	ND	282.3
Dodecyl gallate	1166-52-5	ND	338.4
5-*t*-Butyl-4-hydroxy-2-methyl-phenyl sulfide	96-69-5	ND	358.6
1,3,5-Tris(4-*t*-butyl-3-hydroxy-2,6,-dimethylbenzyl)isocyanurate	40601-76-1	ND	699.9
N-(4-Hydroxyphenyl)stearamide	103-99-1	ND	375.6
2,2´-Methylenebis(4-methyl-6-*t*-butylphenol)	119-47-1	ND	340.5
Bisphenol A	80-05-7	1/10,000	228.3
Bis(4-hydroxyphenyl)methane	620-92-8	1/9,000	200.2
2,2'-Ethylidene bis(4,6,-di-*t*-butyl)	35958-30-6	ND	438.7
Nordihydroguaiaretic acid	500-38-9	Submax	302.4
1,3,5-Trimethyl-2,4,6-tris (3,5-di-*t*-butyl-4-hydroxybenzyl)benzene	1709-70-2	ND	775.2
4,4'-Methylenebis(2,6,-di-*t*-butylphenol)	118-82-1	ND	424.7
Butylated hydroxytoluene	128-37-0	Submax	220.4
2,6-Di-*t*-butylphenol	128-39-2	Submax	206.4
4-*t*-Butylphenol	98-54-4	1/3,000,000	150.2
Catechol	120-80-9	ND	110.1
4-*t*-Butylcatechol	98-29-3	1/300,000	166.2
Butylated hydroxyanisole	25013-16-5	Submax	180.3
Purpurogallin	569-77-7	ND	220.3
Vitamin E	59-02-9	ND	430.8
2,6-Di-*t*-butyl-4-(dimethylamino-methyl)phenol	88-27-7	ND	263.4
Octadecyl-3-(3'5'-di-*t*-butyl-4-hydroxyphenyl)propionate	2082-79-3	ND	530.9
4,4'-Dihydroxybiphenyl	92-88-6	1/9,000	186.2
Tetrabromobisphenol A[a]	79-94-7	ND	543.9
2,4,5-Trihydroxybutyrophenone	1421-63-2	ND	196.2
4-*t*-Octylphenol	140-66-9	1/5,000	206.4

Abbreviations: CAS, Chemical Abstracts Service; MW, molecular weight; ND, not detected; Submax, submaximal response curve.
[a]This compound is a fire retardant.

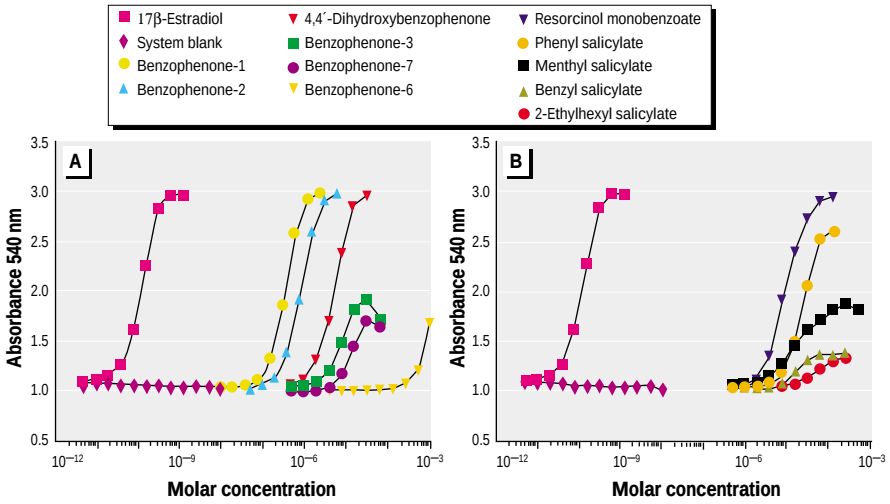

Figure 1. Response of the estrogen yeast bioassay to phenolic additives used as sunscreens and UV inhibitors. Each data point was the mean of triplicate values, and the data are representative of the overall experimental results.

al. (*16*) in a recent study of the estrogenicity of 18 benzophenone derivatives using a recombinant yeast assay. Schultz et al. (*16*) attempted to predict the level of estrogenicity using certain structural rules, but few of the compounds they examined are in use as additives. The most active compounds detected in our own work (i.e., benzophenone-1 and benzophenone-2) have phenolic -OH groups in both *para* and *ortho* positions, whereas 4,4´-dihydroxybenzophenone, having only *para* -OH groups, is surprisingly less active. Other benzophenones either produced no response or were classified as slightly estrogenic due to submaximal response curves, but HPLC with UV detection suggests that trace contamination with benzophenone-1 may be responsible for the slight activity observed in some of these compounds. These other compounds only had phenolic -OH groups in the *ortho* position.

The paraben esters, which have previously been studied in detail using the same yeast assay (*17*), display a progressive increase in estrogenic activity as the molecular weight increases from 152.2 to 228.2. In general, their activity was substantially greater than the salicylate esters, which is what would be expected because the ester group in the salicylate esters is in an *ortho* position relative to the phenolic -OH group. The most active of the salicylate esters studied was phenyl salicylate, which with a molecular weight of 214.2 was close to the mean value for the most active group of compounds, whereas its activity level was close to that of ethyl paraben.

This study suggests that a surprisingly large number of chemicals in everyday use may possess weak estrogenic activity, at least *in vitro*. This contention is supported by a preliminary announcement by Tong et al. (*18*) of a very intelligent and thorough SAR-based modeling study of the 57,000 chemicals in the database of the U.S. Food and Drug Administration. The authors suggest that over 3,000 of the 57,000 chemicals probably possess weak estrogenic activity (at least *in vitro*). The identification of chemicals

Table 6. Estrogenic potency values (10% response level) for compounds displaying submaximal responses.

Compound	CAS registry no.	Estrogenic potency
Benzophenone-3	131-57-7	1/100,000
Benzophenone-6	131-54-4	1/20,000,000
Benzophenone-7	85-19-8	1/300,000
Benzyl salicylate	118-58-1	1/600,000
Menthyl salicylate	89-46-3	1/200,000
Ethylhexyl salicylate	118-60-5	1/2,000,000
Nordihydroguaiaretic acid	500-38-9	1/600,000
Butylated hydroxytoluene	128-37-0	1/8,000,000
2,6, Di-*t*-butylphenol	128-39-2	1/20,000,000
Butylated hydroxyanisole	25013-16-5	1/2,000,000

CAS, Chemical Abstracts Service.

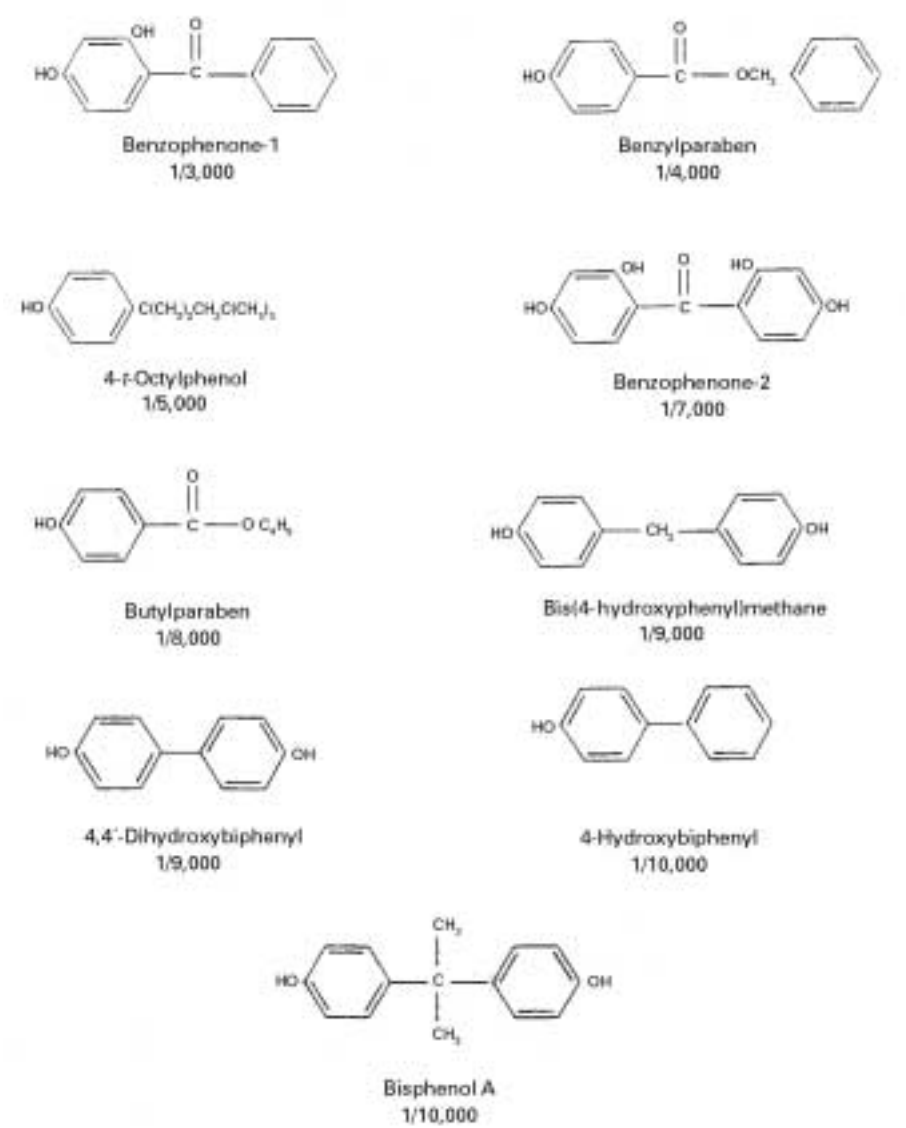

Figure 2. Chemical structures of compounds with an activity relative to 17β-estradiol of 1/1,000 to 1/10,000.

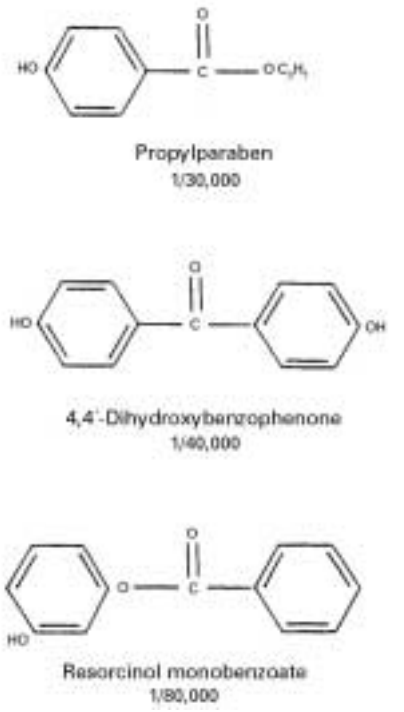

Figure 3. Chemical structures of compounds with an activity relative to 17β-estradiol of 1/10,000 to 1/100,000.

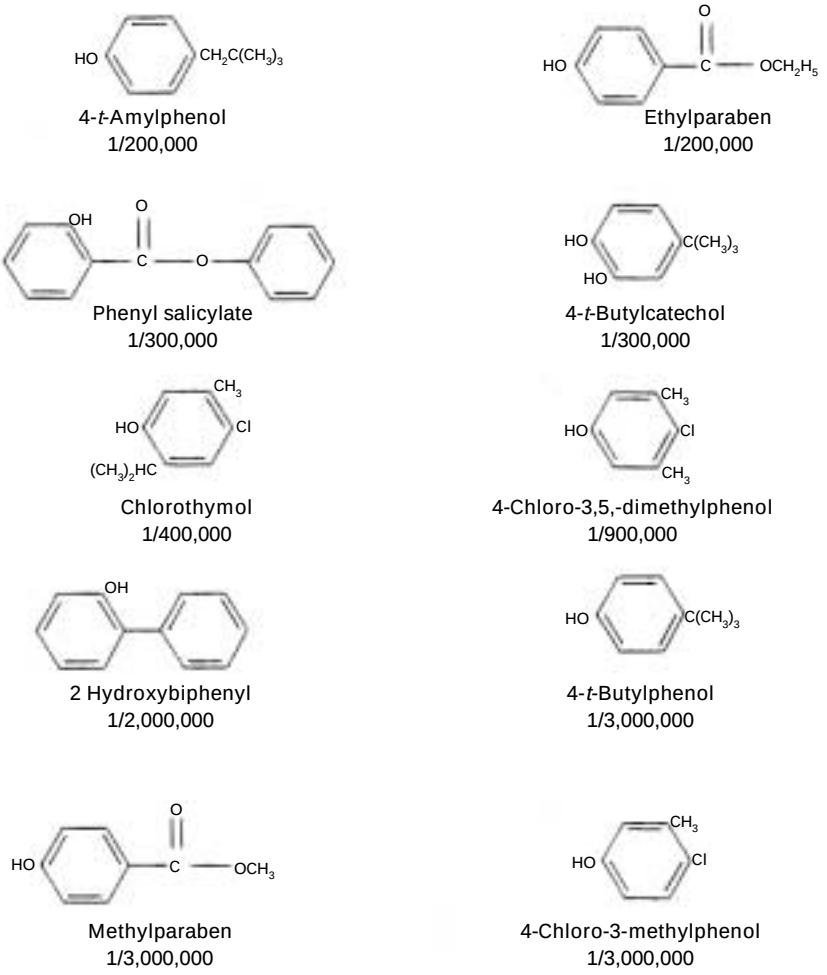

Figure 4. Chemical structures of compounds with an activity relative to 17β-estradiol of < 1/100,000.

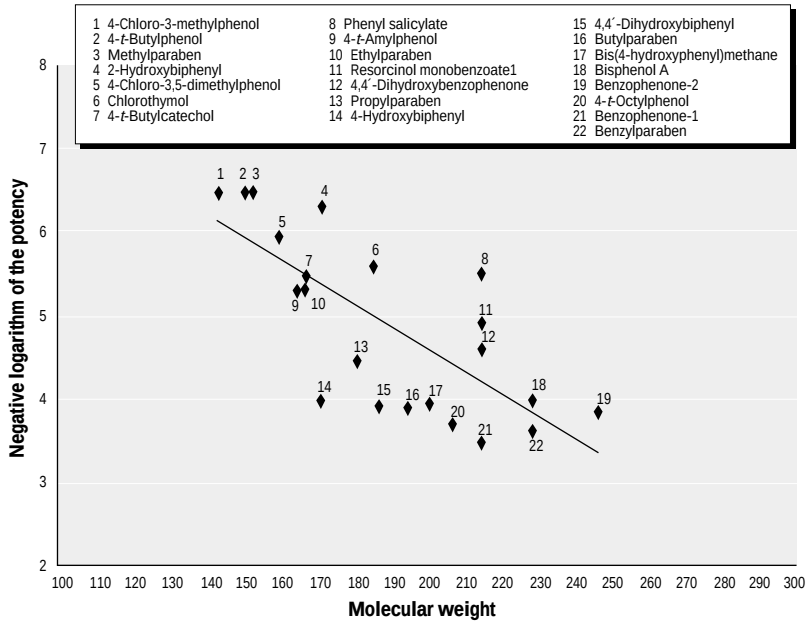

Figure 5. Scatter diagram showing the relationship between molecular weight and estrogenic activity for phenolic additives.

possessing estrogenic activity is, unfortunately, only the initial step (and probably the easiest step). This hazard assessment should lead to risk assessment. To complete realistic risk assessments, it is necessary to know whether the *in vitro* activity (identified here) translates into *in vivo* activity, and if it does, to what degree. It is also necessary to know the extent and route of exposure of the active chemicals. Too often the necessary information required to conduct a meaningful risk assessment is incomplete. A reasonable number (perhaps a few hundred) of chemicals have been shown to possess weak estrogenic activity *in vitro* using a variety of assays. In a few cases, some of these chemicals have recently been investigated *in vivo*, sometimes using relatively short assays, but occasionally using multigenerational studies. For example, the initial demonstration that some phthalates possessed weak estrogenic activity *in vitro* (*5*) stimulated several research groups to investigate the possible endocrine activity of such chemicals *in vivo* (*19*,*20*). Although it is currently difficult to draw firm conclusions, the general message seems to be that chemicals are less active, but nevertheless are often still active, *in vivo* than *in vitro*, when the chemicals are administered orally. However, too few chemicals have been thoroughly tested *in vivo*, using appropriate assays and end points and realistic routes of exposure, to know the extent that endocrine activities and potencies *in vitro* will be manifested *in vivo*. The results mean that humans are exposed to many different (usually weakly) estrogenic chemicals via a number of routes. They lead to the conclusion that there is a strong need to understand the interactive effects of such mixtures with endogeneous estrogens.

Conclusions

This study has shown that a substantial number of phenolic additives incorporated in a group of different products display slight estrogenic activity when assessed by an *in vitro* yeast assay. The strongest activity is chiefly displayed by those compounds in which a phenolic -OH group is in a *para* position to some other substituents and the molecular weight falls within the range 200–250.

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