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# Microbiological methods for the detection of mutagens and cytotoxic components in wood drying condensates from Douglas fir, red oak, southern yellow pine, yellow poplar and eastern white pine[†]

John T. Singer[a,b,*], Jennifer A. Jackson[a,1], Robert W. Rice[b,c]

[a]*Department of Biochemistry, Microbiology and Molecular Biology, University of Maine, 5735 Hitchner Hall, Orono, ME 04469-5735, USA*
[b]*Maine Toxicology Institute, Orono, ME, USA*
[c]*Department of Forest Management, University of Maine, 5755 Nutting Hall, Orono, ME 04469-5755, USA*

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**Abstract**

Three microbiological methods of testing for genotoxic chemicals in uncharacterized wood drying condensates were compared. A commercially successful version of the SOS Chromotest, carried out in microtiter plates, was compared with the original assay that was modified to be performed in soft agar overlays. While the microtiter plate version was quantitative and could be read by an automated microtiter plate reader, the soft agar overlay adaptation was at least six times more sensitive with control chemicals, and was more easily carried out. Each version of the test was able to detect cytotoxic effects of wood drying condensates, but neither test was sensitive enough to routinely detect unknown mutagenic components present in wood drying condensates. The Ames *Salmonella*/microsome assay, while more laborious, revealed cytotoxic dose–response relationships with wood drying condensates from yellow poplar, Douglas fir, white pine, and southern yellow pine; but none of the condensates was mutagenic in the absence of metabolic activation by mammalian liver enzymes. In the presence of rat liver S-9 extracts, yellow poplar condensate resulted in the highest single mutagenic response, yielding over 5,300 revertants/ml of condensate, but was mutagenic for only one of the four Ames test strains. Douglas fir and white pine condensates were mutagenic for three of four Ames strains, and exhibited significant cytotoxicity in the presence and absence of S-9 extract. Southern yellow pine condensate was mutagenic for two of the four Ames strains, but was

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* Corresponding author. Phone: 1 (207) 581-2808; fax: 1 (207) 581-2801.

[1] Present address: Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115-6084, USA.


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far less mutagenic and cytotoxic than condensates from Douglas fir or white pine. Condensate from red oak was cytotoxic, but not mutagenic. The failure of the SOS Chromotest to detect mutagenic components in wood drying condensates was unexpected since over 80% of all compounds tested thus far give similar results in both the Ames test and SOS Chromotest. It appears that mutagenic components present in wood drying condensates are poor inducers of DNA repair, and are not detected by the SOS Chromotest or similar tests that rely on SOS induction.

*Keywords:* Ames test; Environmental mutagen; SOS Chromotest; SOS induction; Wood drying condensates

## 1. Introduction

Most applications for wood require that it be kiln dried. In the United States about $2 \times 10^{10}$ kg of water and volatile organics are removed from nearly 40 million cubic meters of wood annually [1]. Until recently, most of these kiln emissions were vented to the atmosphere. In an effort to recover substantial amounts of wasted energy, dehumidifier dryers employing heat pump technology are being installed. While these dryers can recover up to 50% of the energy lost from a conventional dry kiln, they result in the condensation of massive amounts of water vapor; 20,000 kg of condensate per charge of dried wood is not uncommon. Wood drying condensates contain a 2–3% organic load that consists largely of products that are only broadly defined [2]. Frequently, these condensates flow unregulated into the soils surrounding the dry kilns. Neither the possible benefits of these unknown organics, nor their potential environmental or health risks have been examined.

Methods for detecting potential health risks associated with kiln emissions have not been established, and very few reports exist describing potentially hazardous components in vapor emissions or condensates. All of the data available to date pertaining to wood drying emissions has come either from studies that employed purified test components, or from studies where condensates were generated under extreme conditions that were far removed from those that might be encountered in the field. Ames testing with purified $\alpha$- and $\beta$-pinene, the major monoterpenes released during wood drying, has shown that these compounds are cytotoxic, but not mutagenic [3]. Ames testing with purified 3-carene, a wood-derived monoterpene, resulted in $2.8–4.0 \times 10^4$ revertants/ml when tested in the absence of metabolic activation by S-9 microsomal extracts [4]. Ames testing with actual spruce and birch wood chip drying fumes generated at 170°C demonstrated both mutagenicity and cytotoxicity in the presence of S-9 extracts, but when fumes were condensed, acidified, and extracted with organic solvents, no mutagenicity was recovered in the extract [4].

Because of the recent interest regulatory agencies have taken in determining exactly what risks are and are not associated with wood drying condensates, and because of the regional importance of the forest products industry to Maine, we

first developed laboratory methods that we believe recover volatile organics in wood drying condensates generated at the upper limit of kiln-drying temperatures, and then compared three microbiological methods for the detection of toxic and mutagenic components in condensates from five of the commercially most important species of wood.

Prior to testing, we identified the SOS Chromotest as likely being the most suitable test for the routine screening of large numbers of condensate samples. The SOS Chromotest has been used in testing more than 700 chemicals, and results can be compared to those obtained with the Ames test for over 450 compounds [5]. Eighty-two percent of these compounds gave similar results in both tests, and in cases where discrepancies were apparent, many of the compounds were, in fact, either very weak mutagens or very weak SOS inducers. Therefore, it is likely that many of the reported discrepancies between the tests may actually be the result of differences in interpretation of the test results, rather than differences in the results themselves [5].

The SOS Chromotest [6,7] was designed to detect environmental mutagens based upon their ability to induce SOS DNA repair activities in *Escherichia coli*. Estimates suggest that more than 90% of all environmental mutagens are detectable by induction of SOS repair [8]. There are, however, still compounds such as benzidine, cyclophosphamide, acridines, and ethidium bromide that are mutagenic in assays such as the Ames test, but that fail to result in SOS induction. Conversely, and to a lesser extent, there are compounds such as quinoline-1-oxide that are clearly SOS inducers, but that do not test positive by the Ames test. Thus, while the SOS Chromotest can detect over 90% of all environmental mutagens, additional tests are still necessary to re-evaluate any negative samples, and to assign a relative level of mutagenicity to compounds that test positive via SOS repair induction [6,8].

The SOS Chromotest relies on an increase in $\beta$-galactosidase activity mediated by SOS induction of a chromosomal LexA-regulated *sfiA-lacZ* fusion [6]. The success of the SOS Chromotest has resulted in the development of similar assays that rely on SOS induction of *recA-lacZ* fusions in *E. coli* [9] and *umuC-lacZ* fusions in *Salmonella typhimurium* [10]. All of these assays rely on the same induction pathway and exhibit similar sensitivities [5]. The resulting increase in $\beta$-galactosidase activity upon exposure to a DNA-damaging agent can be detected by monitoring hydrolysis of *o*-nitrophenyl-$\beta$-D-galactopyranoside (ONPG) at 420 nm [7] or 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (X-Gal) at 615 nm [5]. ONPG and X-Gal hydrolysis is so sensitive that the assay can be run in as small a volume as 100 $\mu$l, and can be carried out in 96-well microtiter plates. The results of each well can then be read and recorded by an automated microtiter plate spectrophotometer. The SOS inducing potency (SOSIP) of any unknown sample can then be calculated as $10 \times (\Delta A/\mu$g-, nmol-, or $\mu$l of test compound), relative to that of a known standard such as 4-nitroquinoline-1-oxide (4NQO), which has an SOSIP of 70/nmol [6–8]. The strain of *E. coli* used in the SOS Chromotest also carries a constitutive alkaline phosphatase mutation (PhoC), so that cytotoxicity of unknown samples can be detected as a decrease in the

hydrolysis of *p*-nitrophenyl phosphate (PNPP) at 420 nm [6]. The assay is also amenable to pre-incubation of the sample with mammalian S-9 liver microsomal extracts to test for metabolic activation of promutagens to compounds that are capable of causing DNA damage following the action of oxidative mammalian liver enzymes.

In spite of the apparent suitability of the SOS Chromotest for screening purposes, the Ames *Salmonella*/microsome assay system is still the standard by which newer tests are judged. Because of the ease with which very large populations of genetically identical bacteria can be manipulated, the sensitivity at which one can detect a single mutational event within a population is also quite high. Using the Ames test, one can detect a single prototrophic revertant among $2–4 \times 10^8$ $His^-$ auxotrophs on a single petri plate. Each of the four commonly employed Ames test strains carries a specific point mutation, a single base-pair substitution-, deletion-, or addition, in a gene required for the biosynthesis of L-histidine. Thus, all of the Ames strains are histidine-dependent auxotrophs, and require exogenously added histidine for growth and to form visible colonies on laboratory media. In each case, the exact molecular nature of the $His^-$ mutation has been determined by DNA sequencing. Since all of the $His^-$ auxotrophs carry only point mutations, they each spontaneously revert to the wild-type DNA sequence at a characteristic frequency that can be measured by plating a population of $His^-$ auxotrophs on bacteriological medium lacking histidine. Under these conditions, only revertants carrying an exact reversal of the original $His^-$ mutation will grow and form colonies. The Ames test relies on the fact that agents which are mutagenic increase the reversion frequency above spontaneous levels, and do so in a dose-dependent fashion. Thus, it is possible to assay the mutagenic potency of any unknown compound, or complex mixture of compounds, by monitoring the number of $His^+$ revertants, over and above spontaneous levels, versus the concentration of the uncharacterized test compound [11].

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*E. coli* PQ37 ($F^-$ *thr leu his-4 pyrD thi galE galK* or *galT lacDU169 srl300*::Tn*10 rpoB rpsL uvrA rfa trp*::Muc$^+$ *sfiA*::Mud(Ap, *lac*)cts) [6] was included in the SOS Chromotest Blue kit purchased from Beak Consultants Ltd. (see below). *Uvr* and *rfa* mutations were verified as described below for the Ames test strains. Frozen stocks of verified clones were stored at −85°C in L-broth [12] containing 20% glycerol. *E. coli* PQ37 was grown with shaking at 37°C in L-broth or in Growth Medium A consisting of 0.25% Casitone, 0.25% Yeast Extract, and 0.85% NaCl [5].

Salmonella typhimurium LT2 strains 97a [11,13], 98 [11], 100 [11], and 102 [11,14,15] were obtained from Bruce N. Ames (Division of Biochemistry and Molecular Biology, 401 Barker Hall, University of California, Berkeley, CA

94720, USA) along with complete documentation for their use. The standard set of test strains included TA97a (*hisD6610 rfa ΔuvrB* pKM101) and TA98 (*hisD3052 rfa ΔuvrB* pKM101), strains designed to detect mutagens that induce single base-pair additions or deletions in G-C-rich regions, resulting in a frameshift mutation [11,14]; TA100 (*hisG46 rfa ΔuvrB* pKM101), a strain designed to detect single base-pair substitutions occurring at G-C base pairs [14]; and TA102 (*hisG428 rfa* $Uvr^+$ pKM101/pAQ1-*hisG428*), a DNA repair-proficient strain designed to detect single base-pair substitutions at A-T base pairs arising chiefly as a result of oxidative damage to A. This strain contains about 30 copies of *hisG428* carried on the multicopy plasmid pAQ1 ($Tc^r$), in addition to the chromosomal copy of *hisG428* [14,15]. Each of the four Ames test strains also harbors pKM101 ($Ap^r$), carrying genes *mucAB* that are responsible for the increased sensitivity of the Ames test strains to UV and chemical mutagenesis [16,17].

Verification of the genotypes of the Ames test strains prior to use was performed on ten isolated colonies from each strain as recommended [11]. Briefly, *his* mutations were confirmed by streaking clones on minimal glucose plates containing Vogel-Bonner salts, 1.5% agar, 0.4% glucose and 5 μM biotin with or without 500 μM L-histidine. The deep rough mutation *rfa* was confirmed by crystal violet disc sensitivity testing. The *uvrB* mutation in strains TA97a, TA98 and TA100 was confirmed by cross streaking clones in parallel on a nutrient agar plate, followed by irradiation of sectors of the plate for 0, 1, 3, 6, 9, and 12 s with a 15 W germicidal UV lamp at 0.4 $J/m^2/s$. Strain TA102 served as the $UV^r$ $Uvr^+$ control. The presence of pKM101 (*mucAB*) and pAQ1 (*hisG428*) was confirmed by streaking clones on fresh minimal glucose-histidine plates containing ampicillin (25 μg/ml) and tetracycline (2 μg/ml), respectively [11]. Verified strains were preserved by freezing at −85°C in L-broth containing 20% glycerol.

*S. typhimurium* LT2 strains for Ames testing were cultured in 1% Oxoid Nutrient Broth No. 2 (Unipath–Oxoid Division, P.O. Box 691, Ogdensburg, NY 13669, USA)–0.5% NaCl at 37°C with shaking [11].

### 2.2. The SOS Chromotest

The SOS Chromotest, developed and patented by the CNRS and Pasteur Institute [6,7], is licensed to Orgenics Ltd. (P.O. Box 360, Yavne 70650, Israel, (+972) 8–438754, fax (+972) 8–438758). We purchased the SOS Chromotest Blue kit from the North American distributor, Beak Consultants Ltd. (14 Abacus Road, Brampton, Ontario, Canada L6T 5B7, (+1-416) 794–2325, Fax (416) 794–2338). The kit contained growth medium, lyophilized *E. coli* PQ37, 10% dimethylsulfoxide (DMSO)–saline diluent, genotoxic standards 4NQO and 2-amino anthracene (2AA), an X-Gal-containing blue chromogen solution, PNPP and PNPP diluent, reaction stop solution, and detailed instructions for kit use. User-supplied reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and included glucose-6-phosphate (G6P), NADP, KCl, $MgCl_2$, and Tris base. S-9 liver microsomal preparations from Aroclor-1254-treated rats were

purchased from Microbiological Associates (9900 Blackwell Road, Rockville, MD 20850, USA) and were tested as recommended prior to use [11].

For the quantitative microtiter plate based form of the SOS Chromotest, blue indigo formation from X-Gal hydrolysis was measured at 615 nm and *p*-nitrophenol formation from PNPP hydrolysis was measured at 405 nm using a Flow Laboratories Titertek Multiskan Plus ELISA plate reader (ICN Biomedicals, Inc., 3300 Hyland Avenue, Costa Mesa, CA 92626, USA). Negative controls consisted of a set of quadruplicate wells containing 10 $\mu$l of 10% DMSO-saline containing no sample; positive controls consisted of a quadruplicate set of wells containing serial two-fold dilutions of stock 4NQO (10 $\mu$g/ml) or 2AA (100 $\mu$g/ml; in the presence of S-9 mix) in 10% DMSO-saline. Quadruplicate experimental assays were performed and a typical well without S-9 activation contained 10 $\mu$l of sample or standard in 10% DMSO-saline, and 100 $\mu$l of a freshly grown *E. coli* PQ37 culture diluted to an $A_{600}$ of 0.05 in Growth Medium A. Cells and sample were incubated for 2 h at 37°C followed by the addition of 100 $\mu$l of the X-Gal containing blue chromogen solution and 100 $\mu$l of PNPP solution to 2 wells each of the quadruplicate set of wells. Incubation was continued for 90 min at 37°C, followed by the addition of 50 $\mu$l of stop solution. Well contents were mixed briefly and read on the ELISA plate reader. Normally, two-fold dilutions of condensate and standards (4NQO and 2AA) ranging from undiluted to 1:256 were tested.

For assays that included S-9 activation, the S-9 mix contained (per 5 ml): 100 $\mu$l of 1.65 M KCl–0.4 M $MgCl_2$, 50 $\mu$l of 0.5 M G6P, 100 $\mu$l of 0.1 M NADP, 2.5 ml of 0.2 M Tris-HCl (pH 7.4), 2.05 ml of distilled water, and 200 $\mu$l of S-9 extract [6]. The bacterial cell suspension added to samples receiving S-9 activation contained (per 10 ml): 1 ml of a fresh *E. coli* PQ37 culture in Growth Medium A at an $A_{600}$ of 0.5, 2.5 ml of the above S-9 mix, and 6.5 ml of fresh Growth Medium A. One hundred microliters of the cell suspension–S-9 mix were added per 10 $\mu$l of sample or standard in 10% DMSO-saline, and the remainder of the assay was performed as described above. For each concentration of standards, sample, and for the negative controls in the SOS Chromotest the microtiter plate reader was set to record the results from duplicate wells containing X-Gal and duplicate wells containing PNPP. The average of the $A_{615}$ and $A_{405}$ readings for the negative controls containing 10% DMSO-saline was then subtracted from the average of the duplicate $A_{615}$ and $A_{405}$ readings for 4NQO and 2AA controls and for the experimental wells containing condensate.

### *2.3. The SOS Chromotest spot test*

An alternative to the quantitative microtiter plate based SOS Chromotest is a rapid, qualitative test that can be adapted to be run in soft agar overlays much as the Ames test. Three advantages of this adaptation include: (i) its simplicity, (ii) the fact that cells are exposed to the test compound continually for 16–18 h prior to visual scoring, and (iii) the fact that S-9 preparations are often stabilized by inclusion in soft agar [7]. In this assay, 10-$\mu$l samples of unknowns or standards in

10% DMSO-saline were added to sterile 0.5-cm diameter antibiotic assay discs, which were placed on the surface of soft agar overlays containing *E. coli* PQ37 and X-Gal. S-9 mix was incorporated into the overlay as desired, and up to seven discs containing controls, genotoxic standards, and condensate dilutions were used per plate.

Spot test plates contained M63 minimal salts with 1.5% Bacto agar supplemented with (per liter): 2 ml of 20% lactose, 0.5 ml of 20% glucose, and 2 ml each of 1% solutions of L-tryptophan, L-threonine, L-histidine, uracil, and thiamine. Ampicillin was included at 20 $\mu$g/ml and X-Gal at 40 $\mu$g/ml. Top agar contained 0.8% agar and 0.85% NaCl. Glucose was omitted from spot test plates if S-9 mix was to be included in the top agar overlay [7]. S-9 mix consisted of (per 10 ml): 200 $\mu$l of 1.65 M KCl–0.4 M $MgCl_2$, 50 $\mu$l of 1 M G6P, 150 $\mu$l of 0.1 M NADP, 2.5 ml of 0.4 M Tris-HCl (pH 7.4), 6.1 ml of L-broth, and 1 ml of commercial S-9 extract [7].

Cultures of *E. coli* PQ37 to be used for the spot test were grown overnight in L-broth containing ampicillin (20 $\mu$g/ml), diluted back 1:50 the next morning in fresh medium, and were grown to a final $A_{600}$ of 0.5. One hundred microliters of cell suspension were mixed with 0.4 ml of S-9 mix (or 0.4 ml of L-broth) and 2.5 ml of molten top agar were added, mixed, and immediately poured over a supplemented M63 minimal agar plate. After overlays solidified, antibiotic assay discs were added and 10-$\mu$l samples to be tested were immediately added to the discs. Plates were incubated for 18–24 h at 37°C prior to visual inspection for blue indigo formation around sample discs. All controls and experimental determinations were performed in duplicate.

### 2.4. Ames testing

Overnight (~12 h) cultures of *Salmonella* tester strains were grown in 125-ml baffled-bottom flasks with shaking in 10 ml of Oxoid nutrient broth medium. Cells were concentrated by centrifugation at room temperature in a table-top clinical centrifuge, were suspended to an $A_{600}$ of 4.0 in fresh growth medium, and were used immediately. One hundred microliters of the test strain and 100-$\mu$l dilutions of the test condensate or control were added to 2 ml of 43°C top agar, vortexed, and poured over minimal glucose plates containing Vogel-Bonner salts, 1.5 percent Bacto-agar, and 0.4 percent glucose [11]. Top agar used in the test contained 0.6% Bacto-agar, 0.5% NaCl, 50 $\mu$M L-histidine, and 50 $\mu$M biotin. To compensate for the volume increase that would be encountered when testing $\geq$100 $\mu$l of condensate, concentrations of all top agar components were doubled, and the volume used was halved, when testing 1 ml of undiluted wood drying condensates. All assays including negative controls, positive genotoxic controls, and condensate dilutions were performed in triplicate. Each set of assays was repeated on at least two separate occasions if a dose–response was absent, and on three separate occasions if a dose–response curve was evident.

To simulate the oxidative action of mammalian liver enzymes, condensates were also activated by pre-incubation with S-9 rat liver homogenates. For S-9

activation, the S-9 mix contained 33 mM KCl, 8 mM $MgCl_2$, 5 mM G6P, 4 mM NADP, 100 mM sodium phosphate buffer (pH 7.4), and 1 ml of the commercial S-9 extract per 25 ml of S-9 mix. In a pre-incubation step, 100 $\mu$l of bacterial suspension, 0.5 ml of S-9 mix, and the test sample were held for 20 min at 37°C. Two milliliters of top agar were added, the tubes were vortexed, and the contents were poured over minimal glucose plates. Again, the concentrations of top agar components were doubled and the volumes halved when 1 ml of condensates was tested.

Plates were incubated for 48 h at 37°C and $His^+$ revertant colonies were counted. Each sample dilution and all controls were tested in triplicate, and each condensate was assayed on at least two occasions. For each assay, the average number of induced $His^+$ revertants per plate minus the average number of spontaneous $His^+$ revertants per plate was recorded for each concentration of sample. A dose–response curve of induced revertants per plate versus concentration of the test agent was constructed to determine if a dose–response relationship existed between mutation frequency and a given test agent's concentration [11].

### 2.5. Control mutagens

Recommended control mutagens were used to verify that the Ames test strains were within prescribed limits for spontaneous and induced reversion frequencies, and that *E. coli* PQ37 behaved as expected [7,11]. Methyl methanesulfonate was used to verify the dose response phenotype of strains TA97a, TA100, and TA102. 4-Nitroquinoline-1-oxide was used to verify the dose responses of TA98, TA100, TA102, and *E. coli* PQ37. Sodium azide was used with TA100, and 4-nitro-*o*-phenylene-diamine was used with TA97a and TA98. 2-Aminofluorene and 2-aminoanthracene, control mutagens that require S-9 metabolic activation, were used in the presence of S-9 extract as positive control mutagens for all five bacterial strains. 2-Aminoanthracene was supplied for use with the SOS Chromotest [7] and 2-aminofluorene was recommended and generally used for Ames testing [11]. Negative controls for all strains included 100 $\mu$l of 10% DMSO in normal saline. All chemicals were of the highest grade available and were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO).

### 2.6. Wood drying condensates

Red oak (*Quercus rubra* L.), yellow poplar (*Liriodendron tulipifera* L.), southern yellow pine (*Pinus* spp.), eastern white pine (*Pinus strobus* L.), and Douglas fir (*Pseudotsuga menziesii* (Mirb.)) were all obtained from the United States. Green samples were taken as mill-run lumber or freshly machined chips, and contained both heartwood and sapwood with bark and wane removed. Eastern white pine and red oak were from central Maine, yellow poplar from Tennessee, Douglas fir from coastal Washington state, and southern yellow pine

from Arkansas. The green samples were wrapped in double protective layers of plastic and frozen at −20°C until extraction.

Condensates were collected from wood chips dried at 120°C, the upper limit for kiln drying, in a closed laboratory extraction-drying system. The condensate flask was packed in ice and samples were run until dry. Condensates were sealed and stored at 4°C in dark containers filled to capacity to minimize head space and to prevent loss of volatile components. These condensates were designated 1×, although the actual concentration of organic compounds is likely higher than that found in commercial drying operations due to the higher temperature employed, the lower final moisture content of the dried wood, and the nature of the closed extraction-drying system. For some studies, condensates were further concentrated 1000-fold by lyophilization at −52°C; these condensates were designated 1000×.

## 3. Results

### 3.1. Results of rapid test

Table 1 shows results comparing the SOS microtiter plate test with the spot test. No positive experimental results were detected with the microtiter plate test, even when 10 μl of 1000× condensates were tested. Controls functioned as expected and the limits of detection for 4NQO and 2AA were at or below published values [6–8]. The microtiter plate test also failed to detect cytotoxicity in the form of a decrease in alkaline phosphatase activity, even in wells containing

Table 1
SOS chromotest microtiter plate test and spot test results with wood drying condensates

| Condensate | Microtiter Plate Test | | Spot Test | |
|---|---|---|---|---|
| | −S-9 Mix[a] | +S-9 Mix[b] | −S-9 Mix[a] | +S-9 Mix[b] |
| 1× yellow poplar | − | − | − | − |
| 1000× yellow poplar | − | − | −(T) | −(T) |
| 1× Douglas fir | − | − | − | − |
| 1000× Douglas fir | − | − | −(T) | +(T) |
| 1× red oak | − | − | − | − |
| 1000× red oak | − | − | −(T) | −(T) |
| 1× white pine | − | − | − | − |
| 1000× white pine | − | − | −(T) | −(T) |
| 1× yellow pine | − | − | − | − |
| 1000× yellow pine | − | − | −(T) | −(T) |

The + indicates a positive reaction (SOS DNA repair activities were induced); − is no detectable reaction; (T) indicates a zone of clearing surrounding the discs indicating toxicity effects.

[a] The positive control was 4NQO; limit of detection 0.006 nmol in the microtiter plate assay and 0.001 nmol in the spot test.

[b] The positive control was 2AA; limit of detection was 0.156 μg in both the microtiter plate assay and the spot test.

10 μl of 1000 × condensate. The SOS spot test was the more sensitive test, and a repeatable positive result was obtained with 10 μl of a 1000 × Douglas fir condensate after metabolic activation with S-9 mix (Table 1). Cytotoxicity was also detected as a zone of growth inhibition around all discs containing 1000 × wood drying condensates, but not around discs seeded with 1 × condensates. Results with genotoxic standards indicated that the spot test was more sensitive than the microtiter plate test in the absence of S-9 activation, repeatably detecting just 0.001 nmoles of 4NQO.

### 3.2. Ames test

The Ames test is designed to detect both mutagenicity and cytotoxicity. Mutagenicity is indicated by an increase in the numbers of $His^+$ revertants, over and above spontaneous control levels, while toxicity results in cell death and numbers of $His^+$ revertants below those obtained for spontaneous controls. To determine if there were, in fact, DNA-damaging agents present in the condensates that were not being detected by the SOS Chromotest, the largely negative results obtained above were compared with results obtained by Ames testing. Even 10 μl of a 1000 × condensate had pronounced cytotoxic effects on the Ames test strains (not shown), making a mutagenicity determination impossible in the presence of pronounced toxicity.

To separate toxic effects from mutagenicity, ten-fold serial dilutions of 1 × condensates ranging from 1 μl of a $10^{-6}$ dilution to 1 ml of undiluted condensate were subjected to Ames testing. Table 2 shows that even at concentrations as high as 1 ml/plate, none of the condensates was mutagenic in the absence of S-9 extract, but all condensates exhibited cytotoxic effects. Table 2 shows that, upon activation with S-9 extract (20 μl per plate), however, there were detectable mutagenic dose–response relationships with all condensates tested. Examples of typical dose–response curves for two of the condensates are shown in Figs. 1 and 2.

## 4. Discussion

The above results demonstrate that the SOS Chromotest in microtiter plate and spot test forms failed to detect mutagenic components in wood drying condensates, making it unsuitable for use as a general screening tool for condensates. This result was unexpected in view of the previous successes reported by other investigators using the SOS Chromotest for monitoring a variety of environmental genotoxic agents in groundwater, river water, and sediments [18–20]. No data exists, however, for the use of such tests in monitoring natural products in the non-particulate fraction of wood drying emissions. The above results suggest that natural products present in wood, and given off during drying, are ineffective inducers of SOS DNA repair, and therefore, are likely not subject to detection by any of the popular rapid tests that rely on DNA repair induction mechanisms. In

Table 2
Summary of Ames testing of wood drying condensates

| Condensate | *S. typhimurium* | Results (−)S-9 | Results (+)S-9 |
|---|---|---|---|
| Yellow poplar | TA97a | No effect | Mutagenic |
| | TA98 | No effect | No effect |
| | TA100 | Toxic | No effect |
| | TA102 | Toxic | No effect |
| Douglas fir | TA97a | No effect | Mutagenic and toxic |
| | TA98 | No effect | No effect |
| | TA100 | No effect | Mutagenic |
| | TA102 | Toxic | Mutagenic |
| Red oak | TA97a | No effect | Toxic |
| | TA98 | No effect | No effect |
| | TA100 | Toxic | Toxic |
| | TA102 | Toxic | Toxic |
| White pine | TA97a | Toxic | Mutagenic and toxic |
| | TA98 | No effect | No effect |
| | TA100 | Toxic | Mutagenic and toxic |
| | TA102 | Toxic | Mutagenic and toxic |
| Southern yellow pine | TA97a | No effect | Mutagenic |
| | TA98 | No effect | No effect |
| | TA100 | No effect | Toxic |
| | TA102 | Toxic | Mutagenic and toxic |

Serial ten-fold dilutions of condensates from 1 $\mu$l of a $10^{-6}$ dilution to 1 ml of undiluted condensate were tested in triplicate and on at least two occasions as described in the text.

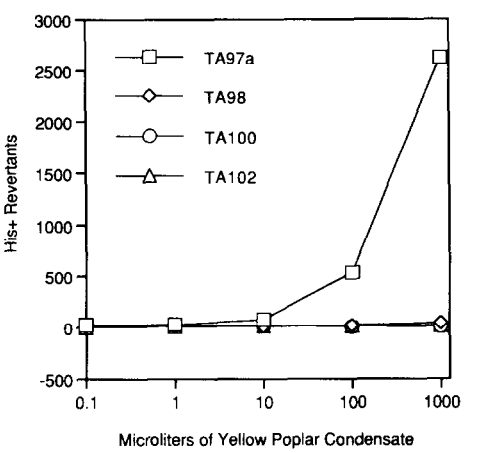


Fig. 1. Ames testing of yellow poplar condensate. All points are the average of triplicate determinations; error bars indicate standard error. The volume of yellow poplar condensate used was plotted on a logarithmic scale in order to separate individual data points. Results for *S. typhimurium* TA97a are indicated by open squares; for TA98 by open diamonds; for TA100 by open circles; and for TA102 by open triangles.

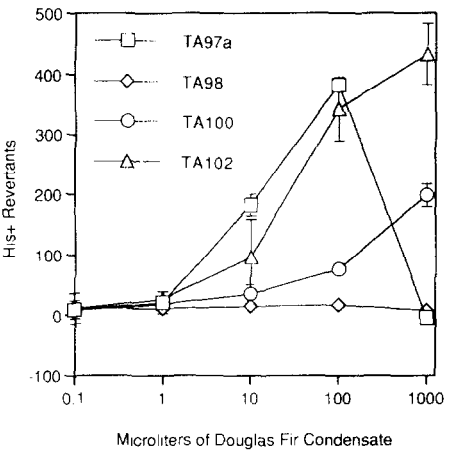


Fig. 2. Ames testing of Douglas fir condensate. All points are the average of triplicate determinations; error bars indicate standard error. The volume of Douglas fir condensate used was plotted on a logarithmic scale in order to separate individual data points. Results for *S. typhimurium* TA97a are indicated by open squares; for TA98 by open diamonds; for TA100 by open circles; and for TA102 by open triangles.

support of this, condensates failed to induce *recA-lacZ* fusions in permeabilized cells of *E. coli* and a marine bacterium, *Vibrio anguillarum*, as well, while 4NQO induced significant levels of $\beta$-galactosidase activity (not shown).

While we were unable to develop a rapid method for screening condensates for genotoxins, our method for isolating aqueous condensate was clearly more direct and more successful than previous attempts by Kurttio et al. [4] that included acidification and solvent extraction. Previously, aqueous condensates produced at unrealistically high temperatures (170°C) were acidified to pH 2.0 with HCl, extracted with dichloromethane, the extract evaporated to near dryness, and the remaining residue dissolved in DMSO. While the heated wood chip drying fumes themselves were shown to be mutagenic in these studies, the aqueous condensate was never tested for mutagenicity, and all mutagenicity was lost from the extract upon solvent extraction of the condensate. The above extraction and drying conditions likely resulted in the loss of low molecular weight volatile organics that were responsible for the original mutagenicity of the wood chip drying fumes [4]. Our method of testing the condensates ensures that loss of volatile organics is minimized. It should be pointed out that while we have designated our initial condensates as '1 ×', these condensates contain 2–6 mg of total organics per milliliter, and are probably significantly more concentrated than natural condensates that would be encountered in the field. We are presently conducting analyses to determine concentration factors for our condensates relative to field-generated condensates.

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## References

[1] Rice, R.W., Howe, J.L., Boone, R.S. and Tschernitz, J. L. (1993) Kiln drying lumber in the United States–a survey of volume, species, kiln capacity, equipment, and procedures, 1992–1993. US Dep. Agric. Forest Serv., Forest Products Laboratory, Gen. Technical Report FPL-GTR-81.

[2] Hillis, W.E. (1987) Heartwood and Tree Exudates, Springer-Verlag, New York.

[3] Florin, I., Rutberg, L., Curvall, M. and Enzell, C.R. (1980) Screening of tobacco smoke constituents for mutagenicity using the Ames test. Toxicology 15, 219–232.

[4] Kurttio, P., Kaliokoski, P., Lampelo, S. and Jantunen, M.J. (1990) Mutagenic compounds in wood-chip drying fumes. Mut. Res. 242, 9–15.

[5] Quillardet, P. and Hofnung, M. (1993) The SOS chromotest: a review. Mut. Res. 297, 235–279.

[6] Quillardet, P., Huisman, O., D'ari, R. and Hofnung, H. (1982) SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K-12 to measure genotoxicity. Proc. Natl. Acad. Sci. USA 79, 5971–5975.

[7] Quillardet, P. and Hofnung, M. (1985) The SOS Chromotest, a colorimetric bacterial assay for genotoxins: procedures. Mut. Res. 147, 65–78.

[8] Quillardet, P., de Bellecombe, C. and Hofnung, M. (1985) The SOS Chromotest, a colorimetric bacterial assay for genotoxins: validation study with 83 compounds. Mut. Res. 147, 79–95.

[9] Nunoshiba, T. and Nishioka, H. (1991) Rec-lac test for detecting SOS-inducing activity of environmental genotoxic substances. Mut. Res. 254, 71–77.

[10] Oda, Y., Nakamura, S., Oki, I., Kato, T. and Shinigawa, H. (1985) Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens. Mut. Res. 147, 219–229.

[11] Maron, D.M. and Ames, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. Mut. Res. 113, 173–215.

[12] Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

[13] Levin, D.E., Yamasaki, E. and Ames, B.N. (1982) A new *Salmonella* tester strain, TA97, for the detection of frameshift mutagens. Mut. Res. 94, 315–330.

[14] Levin, D.E., Hollstein, M., Christman, M.F., Schwiers, E.A. and Ames, B.N. (1982) A new *Salmonella* tester strain (TA102) with AT base pairs at the site of mutation detects oxidative mutagens. Proc. Natl. Acad. Sci. USA 79, 7445–7449.

[15] Levin, D.E., Hollstein, M., Christman, M.F., Schwiers, E.A. and Ames, B.N. (1982) Detection of oxidative mutagens with a new *Salmonella* tester strain (TA102). Methods Enzymol. 105, 249–255.

[16] Langer, P.J., Shanabruch, W.G. and Walker, G.C. (1981) Functional organization of plasmid pKM101. J. Bacteriol. 145, 1310–1316.

[17] Walker, G.C. (1987) The SOS response of *Escherichia coli*. In: *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology (Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M. and Umbarger, H.E., eds.), pp. 1346–1357, American Society for Microbiology, Washington, DC.

[18] Kwan, K.K. and Dutka, B.J. (1992) A novel bioassay approach: direct application of the Toxi-chromotest and the SOS Chromotest to sediments. Environ. Toxicol. Water Qual. 7, 49–60.

[19] Lan, Q., Dickman, M. and Alvarez, M. (1991) Evidence of genotoxic substances in the Niagra River watershed. Environ. Toxicol. Water Qual. 6, 1–15.

[20] Xu, H., Dutka, B.J. and Kwan, K.K. (1987) Genotoxicity studies on sediments using a modified SOS Chromotest. Toxicity Assessment 2, 79–87.