# Detection and Characterization of Potential Carcinogens in Eucalyptus and Chaparral Herbal Teas

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On my honor, I have neither given nor received any unacknowledged aid on this paper.

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Abstract

Four different mutant strains of Salmonella typhimurium were used in accordance with the Ames mutagenicity assay to detect and characterize any mutagenic properties in two common herbal teas: chaparral and eucalyptus. Although statistical verification was not obtained (due to insufficient funding, explained below), the assay shows apparent evidence of the presence of both one or more frameshift mutagens and one or more base-pair substitution mutagens in the eucalyptus tea tested.

## I. Introduction

For several reasons, the group of diseases known as "cancer" is among the most feared of human afflictions. Approximately 600,000 new cases of cancer are reported in the United States each year; according to present trends, one out of every five Americans will die as a direct result of cancer or complications arising from its treatment (11). Other afflictions, especially arterial diseases, tend to affect older men who lead less than healthy lifestyles, whereas cancer seems to indiscriminately strike the old and young, rich and poor, fat and thin (7). The diagnosis of cancer is, in more than half of all cases, a sentence of death. Perhaps the most distressing aspect of cancer, however, is our incomplete understanding of its etiologies and pathogenesis, resulting in relatively ineffective cancer treatment. Development of a treatment or cure is contingent on a much deeper understanding of these two aspects of cancer.

Many aspects of cancer are presently understood. The term "cancer" refers to all types of invasive neoplasms, of which more than 100 have been shown to affect humans. Neoplasia, the formation of localized swellings (tumors), is the process(es) by which an organism's normal controlling mechanisms that regulate cell growth and differentiation are impaired, resulting in uninhibited, progressive cell growth (7). Many agents have been proven to promote the growth of malignant neoplasms; they are termed carcinogens.

It was long suspected that at least some cancer is the result of genetic mutations in cellular DNA. Such has been shown through research to be the case (17). In fact, 85% of tested carcinogens have been shown to be mutagenic, or "genotoxic," to use Dr. Ames's terminology (2). Mutations can either occur as DNA base-pair substitutions, in which

another base pair displaces the proper base pair in the DNA (e.g., an A-T pair displaces a C-G), or as frameshift mutations, in which a base pair (or sequence of base pairs) is either inserted in or deleted from the proper DNA base-pair sequence.

Mutagens that cause base-pair substitutions do so by chemically modifying the proper base-pairs of DNA, resulting in a substitution of one pair by another (e.g., AT replaces CG) (16). This substitution can result in coding for the wrong amino acid and thus a partly-functional or nonfunctional protein.

Most known frameshift mutagens contain flat, aromatic carbon rings which can fit into (intercalate in) the DNA double helix (13). Intercalation can lead to the insertion or deletion of a base-pair, resulting in alteration of the reading frame in translation. This frameshift mutation causes mutations that are more catastrophic to protein function than mere base-pair substitutions, as every 3-base amino acid codon translated after the insertion or deletion is completely changed, thus coding for completely different amino acids. The result usually is a completely nonfunctional protein (17).

The ultimate result of each type of mutation is the synthesis of a partly-functional or nonfunctional protein. Impairment of protein function is a serious matter, since exhaustive evidence supports the hypothesis that the mechanisms governing cell growth depend on certain proteins (enzymes). If these proteins are not fully functional, cell growth is not properly controlled and neoplasia could result.

**Cancer and Food**

Epidemiologists have estimated that 70% of human cancer would be treatable or even preventable if the main risk factors (i.e., the carcinogens involved) were elucidated (4). International epidemiological studies have indicated that variations in exposure to carcinogens in the diet is the most promising area of cancer research (3). Perera and Boffetta (14) note that "there is clearly a need for more research on dietary carcinogens."

All carcinogens are not man-made. Many preservatives, insecticides, and other

artificial chemicals do have mutagenic properties. Mutagenic chemicals, however, have long been known to also exist in small amounts in the vegetables and other plant products we eat. Plants have evolved the ability to produce these antiherbivorous chemicals; they are nature's pesticides. As a result of the recent "back to nature" trend in our society, we eat, for example, the carcinogen canavanine in alfalfa, carcinogenic hydrazines in mushrooms, and several carcinogens in herbal teas (5). Carcinogens are not limited to natural foods, however; for example, theobromine, a potent carcinogen, is present in small amounts in processed chocolate. Nitrates and nitrites (which are converted to carcinogenic nitrosamines and other nitroso derivatives in vivo) are present in processed meats such as bologna and hot dogs. Ames and his associates have published a ranking of the relative potency of several carcinogens, including those just mentioned, which are regularly found in common foods (3).

**The Ames Test**

The identification of potent carcinogens in the environment is necessary but difficult. In addition to the multitudes of chemicals present naturally, an estimated 50,000 different man-made chemicals are currently in use, and between 500 and 1,000 new chemicals are marketed every year (10). The standard rodent tests and corresponding HERP index (human exposure/rodent potency dose) determination are very time-consuming and expensive (4). The need for a quick, inexpensive method of determining a chemical's carcinogenicity is evident.

The correlation between carcinogenicity and mutagenicity is the basis of one of the most valuable quick and inexpensive chemical mutagenicity assays used in cancer research today. The Ames test, developed by Bruce N. Ames, determines whether a potential mutagen is indeed mutagenic (the term used by Ames is "genotoxic") to bacteria (2). The basic premise of the test is if a substance is genotoxic to bacterial DNA, it will be genotoxic to human DNA as well.

The bacteria used in the Ames test are four mutant strains of Salmonella typhimurium. Each strain contains a different mutation in the histidine-synthesizing operon on the DNA; thus, different types of mutagens can be detected. In addition, each strain has been genetically altered to render it more sensitive to the actions of mutagens. The rfa mutation, present in all four strains, causes partial loss of the bacterium's protective capsule, allowing large (potentially mutagenic) molecules entry into the cell and the nucleus. The uvrB mutation, present in strains TA 97, TA 98, and TA 100, is a deletion of a gene which codes for the DNA excision-repair system. This uvrB mutation also involves a deletion of a biotin-synthesizing operon; consequently, these bacteria also require biotin for growth. Each of these strains contains the R-factor plasmid pKM101, which increases mutagenesis by enhancing an error-prone DNA excision repair system already present in these strains. The strain TA 102 also contains the plasmid pAQ1, which carries the hisG428 mutation, which is very sensitive to certain mutagens (11, 16).

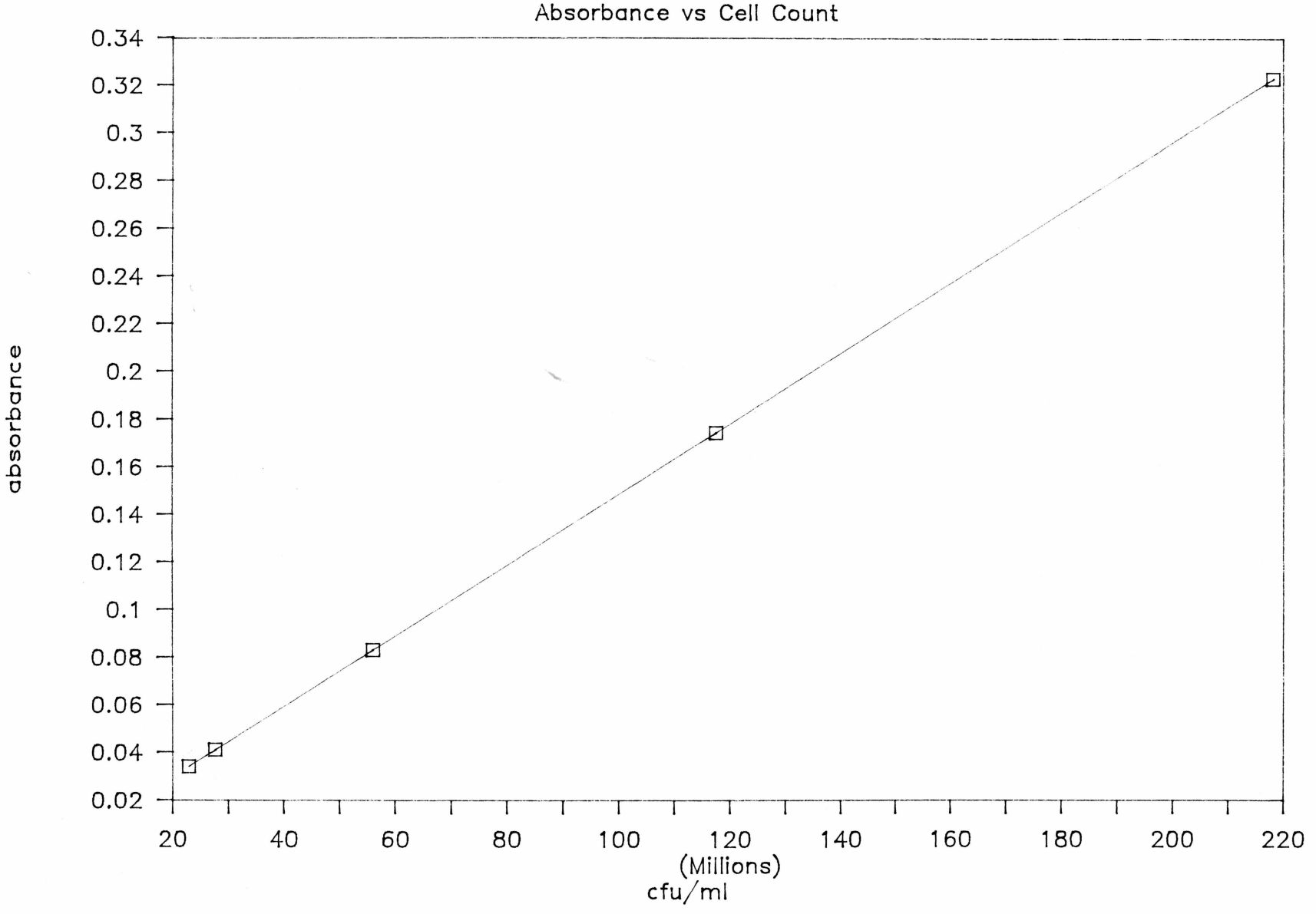
The strain TA 98 detects frameshift mutagens. Its histidine mutation hisD3052 contains 8 repeating CG subunits near a deleted base pair. The mutation renders the enzyme histidinol dehydrogenase non-functional. This mutation is restored to correct order by a base-pair insertion at that site in the DNA, aided by a frameshift mutagen. The strain TA 97 contains the hisD6610 mutation, in addition to a CG run similar to that in the hisD3052 mutation. It also detects frameshift mutagens. The strain TA 100 contains the hisG46 mutation, which alters the gene coding for the first enzyme involved in histidine synthesis. The GAG codon (coding for leucine) in the prototroph DNA is changed to GGG (proline) in the auxotroph, and the enzyme translated is nonfunctional. This mutation is restored to correct order by a base-pair substitution mutation. Finally, the strain TA 102 contains a mutation in the hisG gene which is sensitive to many oxidizing mutagens not detected by the other tester strains (2, 12). TA 102 contains AT base-pairs at the site of the mutation, whereas the other three strains contain CG base-pairs.

In the Ames test, each tester strain is individually mixed and (optionally) preincubated with the mutagen, molten top agar, and an enzyme solution termed the "S9 mix" before plating. The S9 mix activates many promutagens which would otherwise test negative in this assay. The S9's presence in the Ames test is necessary to approximate promutagen oxidation by liver enzymes; in fact, S9 is usually prepared from rat liver tissue, and therefore contains actual mammalian liver enzymes (12). Preincubation removes interfering histidine, if present, in the substance to be tested, and in many cases increases the sensitivity of the assay (1).

The preincubated top agar solution is poured over a minimal medium lacking histidine. After 24-48 hours of incubation at 37C, colonies may appear. These colonies have regained the ability to synthesize histidine -- they have genetically reverted to prototrophy. Reversion of the mutant tester strains to prototrophy indicates mutagenic action at the histidine-coded locus or at some sequence controlling that locus. Each strain has its characteristic average number of spontaneous revertant colonies. A potential mutagen causing significantly more revertants than these averages is determined to be a promutagen or mutagen. This compound would then be tested on rodents to determine information such as its $TD_{50}$ dose (amount causing tumors in 50% of tested rodents) and HERP index value.

The following execution of the Ames mutagenicity assay examines the mutagenic properties, if any, of chaparral and eucalyptus herbal teas. These two teas were chosen for testing because of the presence of alleopathic and herbivore defense compounds in oils they produce. Eucalyptus globulus, a tree, and chaparral, a collection of woody herbs and shrubs (one of which is commonly Adenostoma fasciculatum), are common in Australia and southern California. Both plant types appear to, by means of alleopathic compounds, either completely or partially inhibit the germination or growth of other plants nearby. They also have been shown to produce compounds that deter herbivory by insects and other animals. These two types of compounds have a primary toxic effect, interfering

# Salmonella typhimurium (TA 100)



with some biochemical process in a competing plant or herbivore. Many herbivores and plants, however, have evolved biochemical and other defenses against these toxic compounds. It is suspected, though, that long term exposure to these compounds can have a secondary toxic effect -- mutagenesis. The advantage gained by these plants in having alleopathic and herbivore defense compounds with this secondary toxic effect is evident. By testing these teas with the Ames mutagenicity assay, it is possible to determine if this secondary toxic effect is indeed mutagenic in nature (6, 18).

These two teas were merely screened for possible mutagenicity. These assays are by no means a complete description of the teas' mutagenic properties. The S9 enzyme mix is costly, and due to the confines of an undergraduate honors research budget, the assays below are of the most general and superficial nature. To complete the initial screening process, I would have required on the order of five times the amount of S9 with which I worked. With a proper amount of the S9, enough plates could have been counted so that statistical significance would determine whether a tea is potentially mutagenic and, therefore, potentially carcinogenic. Still, such a superficial screening survey of a small amount of plates can be of some value in determining which foods exhibit suspiciously mutagenic tendencies.

## II. Materials and Methods

### 1. Growing Cultures

Tester strain cultures were grown in glass tubes (not ethylene oxide-sterilized) using Oxoid nutrient broth No. 2 (8) to a density of 1-2x$10^9$ cells/ml . Cell density was determined using turbidity measured by a spectrophotometer at 650 nm (see Figure 1). Difco broth was not used because it has been shown to increase the number of spontaneous revertants in the assay; Oxoid broth was used instead. The absorbance curve thus obtained was used to grow the cultures to the desired density.

Stored isolates of the strains were prepared by adding 0.1 ml sterile glycerol per ml cell culture (grown to 1-2x$10^9$ cells/ml) and freezing, first in a 0C freezer and then in a -70C ultra-low freezer.

The genotypes of each of the four strains were confirmed according to sensitive tests described in the literature (Maron and Ames, 1983: 179-181).

## 2. Spontaneous Reversion

In order to determine the spontaneous revertant colonies per plate for each strain, one control plate (containing everything in the assay except the potential mutagen) was included with each assay. The availability of more S9 would have allowed more reliable determination of the average number of spontaneous revertants per strain.

## 3. Preparation of the S9 Mix

A 500 mg/kg intraperitoneal injection of 200 mg Aroclor 1254 / ml corn oil is administered to male rats 5 days before sacrifice. The rats are allowed to eat and drink their fill until 12 hours before sacrifice, when the food is removed.

After sacrifice, the rat liver is removed aseptically. (In all following steps, the liver is kept chilled at 0-4C.)

1. Homogenize the liver using a Potter-Elvehjem apparatus with a teflon pestle.
2. Centrifuge the homogenate at 9000g for 10 min.
3. Decant and save the supernatant, which contains the S9 enzymes.
4. Immediately freeze the supernatant on a bed of crushed dry ice and then store at -80C.
5. When ready to use the S9, it is thawed at room temperature, and added to the buffered cofactor mix.

The S9 mix used in this research was obtained from Organon-Teknika of Durham, NC. The above process was used in preparing their marketed S9.

## 4. Potential Mutagen Preparation

The two teas used were chaparral leaf tea and Eucalyptus globulus leaf tea, both marketed by Frontier Herbs, Inc. Both were obtained from the Rockbridge Food Co-Op of Lexington, VA. 8.5 g (dry mass; the approximate amount used for a pot of tea) of each tea was added to 100 ml of boiling tap water and allowed to brew for 20 minutes. The resulting tea solutions were then strained, filtered, and filter-sterilized.

## 5. The Mutagenicity Test

Normal assay plates: 10 ml of a sterile solution of 0.5 mM L-histidine - HCl/ 0.5 mM biotin was added to 100 ml of autoclaved top agar and mixed thoroughly. 2 ml of this top agar was then pipetted into a glass test tube at 45C. To this top agar the following was added: 0.1 ml of a fresh overnight culture of one of the tester strains; 0.1 ml of the sterile tea; 0.5 ml of the S9 mix (for each strain, one plate per tea was prepared without S9 to characterize the S9's effect, if any). These components were gently vortexed for 3 seconds, poured onto a minimal glucose agar plate (glass, not ethylene-oxide sterilized), and evenly distributed. For each strain, two normal assay plates were prepared per tea. The top agar was allowed to harden. The plates were inverted and incubated at 37C for 48 hours.

Spontaneous reversion control plates: contained all of the above components except the tea. For each strain, one control plate was prepared per tea. Plates were incubated as above.

Preincubated plates: each potential mutagen was also preincubated to remove any interfering histidine in the tea as described by Aeschbacher (1987) and by Maron and Ames (1983) with the assay components as follows (1, 12):

1. Add 0.1 ml of the potential mutagen in solution (dissolved in water or DMSO), 0.1 ml of fresh tester strain culture, and 0.5 ml S9 mix (or 0.5 ml of salt buffer in the case of an S9 control) to a glass test tube.
2. Vortex gently and incubate, with shaking, at 37C for 20 min.
3. After preincubation, pour the contents of the tube onto a minimal glucose agar plate (the agar again lacking histidine), and the plates incubated as above.

After incubation, the plates were removed and the revertant colonies on each were counted (scored). After the plates are scored, statistical analysis is normally used to determine the significance of the difference in the number of revertant colonies on the potential mutagen-containing plates and the control plates. A t-test is recommended, although other methods are also available (12). In this assay, only a rough comparison between the numbers of colonies in the different plates could be made. With enough S9, an actual dose-response curve could have been formulated for positively mutagenic foods, illustrating the mutagenicity of increasing doses of the mutagen.

III. Results

(SRC refers to spontaneous reversion control)

**Eucalyptus**

| Strain | Plate type | # of revertant colonies |
|---|---|---|
| TA 97 | Normal assay | 307 |
| | Normal assay | 374 |
| | Preincubated | 317 |
| | Without S9 | * |
| | SRC | 177 |
| TA 98 | Normal assay | 87 |
| | Normal assay | 109 |
| | Preincubated | 139* |
| | Without S9 | 19 |
| | SRC | 26 |
| TA 100 | Normal assay | 194 |
| | Normal assay | 175 |
| | Preincubated | * |
| | Without S9 | 66 |
| | SRC | 100 |
| TA 102 | Normal assay | 187* |
| | Normal assay | 200* |
| | Preincubated | 65* |
| | Without S9 | 197 |
| | SRC | 209 |

*the colonies, if present, on these plates were difficult to count, apparently due to either overincubation of the enzyme mix (and resultant protein denaturation) or to

contamination.

**Chaparral**

| Strain | Plate type | # of revertant colonies |
|---|---|---|
| TA 97 | Normal assay | 166 |
| | Normal assay | 173 |
| | Preincubated | * |
| | Without S9 | 79 |
| | SRC | 162 |
| TA 98 | Normal assay | 39 |
| | Normal assay | 30 |
| | Preincubated | 12* |
| | Without S9 | 19 |
| | SRC | 23 |
| TA 100 | Normal assay | 129 |
| | Normal assay | 136 |
| | Preincubated | * |
| | Without S9 | 84 |
| | SRC | 133 |
| TA 102 | Normal assay | 261 |
| | Normal assay | 273 |
| | Preincubated | * |
| | Without S9 | 166 |
| | SRC | 225 |

*plates difficult to read due to possible reasons mentioned above

## IV. Discussion

Each Salmonella typhimurium mutant strain has a characteristic range of spontaneous revertant colonies: TA 97 (90-180 colonies), TA 98 (30-50), TA 100 (120-200), and TA 102 (240-320). The literature points out that these numbers may vary slightly with the addition of S9 (12). All of the spontaneous reversion control values were within or nearly-within these ranges; hence, the controls were acceptable.

The strains TA 97 and TA 98 detect frameshift mutagens. In the eucalyptus results, the average normal assay results (340.5 and 98, respectively) and preincubation results (317 and 139, respectively) indicates an obvious increase in the number of

revertants in the presence of eucalyptus tea relative to the SRC's (177 and 26, respectively). While these results offer no statistical evidence, it does suggest the presence of a frameshift mutagen in eucalyptus tea.

The strain TA 100 detects base-pair substitution mutagens. In the eucalyptus results, the average normal assay result (184.5) also indicates an obvious increase in the number of revertants in the presence of this tea relative to the SRC (100). However, since 184.5 still falls within the range of possible spontaneous revertant colonies for TA 100 (120-200), there remains some doubt as to the presence of a base-pair substitution mutagen. Statistical verification is needed.

The strain TA 102 detects various oxidative and other mutagens not detected by the other three strains. In the eucalyptus results, the average normal assay result (193.5) is both quite close to the SRC value (209), and actually falls below the range of possible spontaneous revertant colonies for TA 102 (240-320). These results would appear to indicate no mutagen detection by TA 102; again, statistical verification is needed.

In the chaparral results, the normal assay results using TA 97 and TA 98 (averages of 169.5 and 34.5, respectively) were slightly higher in the presence of chaparral tea than in the SRC's (162 and 23, respectively). Both normal assay numbers fall within or below the range of possible spontaneous revertant colonies for these two strains. This is not as positive an indication of the presence of a frameshift mutagen as in the eucalyptus results, but still would be a point of further investigation.

The normal assay results using chaparral and TA 100 (average of 132.5) appears to not differ much if at all from the SRC value (133). Chaparral appears to contain no base-pair substitution mutagens, though statistical verification is still needed.

The normal assay results using chaparral and TA 102 (average of 267) is slightly higher than the SRC value (225). 267 still falls within the range of possible spontaneous revertant colonies for TA 102. These results possibly indicate the presence of a weak oxidative mutagen; statistical verification would decide whether the difference between the

normal assay average and the SRC was enough to warrant a significant difference and therefore the detection of a mutagen.

In general, the number of revertant colonies was less on plates without S9 than with it (all except TA 102 - eucalyptus). This indicates the necessity of the S9 mix in reacting in some fashion with the teas in order to activate promutagens (thus approximating the liver's environment).

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