NTP
National Toxicology Program
U.S. Department of Health and Human Services

NTP Technical Report on the 3-Month Toxicity Studies of Estragole (CAS No. 140-67-0) Administered by Gavage to F344/N Rats and B6C3F1 Mice

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National Toxicology Program
Toxicity Report Series
Number 82

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National Institutes of Health
Public Health Service
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FOREWORD

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

The Toxicity Study Report series began in 1991. The studies described in the Toxicity Study Report series are designed and conducted to characterize and evaluate the toxicologic potential of selected substances in laboratory animals (usually two species, rats and mice). Substances selected for NTP toxicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in the Toxicity Study Reports are based only on the results of these NTP studies. Extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection *per se* is not an indicator of a substance's toxic potential.

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Estragole

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Administered by Gavage
to F344/N Rats and B6C3F1 Mice

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NIH Publication No. 11-5966

National Institutes of Health
Public Health Service
U.S. Department of Health and Human Services

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CONTENTS

SUMMARY

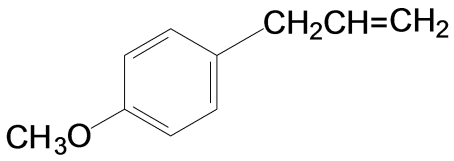
Background
Estragole occurs naturally in a variety of foods and spices such as basil, tarragon, bay leaves, fennel, and anise, and it is used as an additive, flavoring agent, and fragrance in a variety of foods, cleaning products, and cosmetics. It is related in structure to methyleugenol and isoeugenol, which have been shown to cause cancer in rodents. We studied estragole to determine the possible toxic effects on rats and mice after 3 months of exposure.

Methods
We gave groups of 10 male and 10 female rats and mice corn oil solutions containing estragole 5 days per week for 3 months by depositing the solutions directly into their stomachs through a tube. The doses delivered daily were 37.5, 75, 150, 300, or 600 milligrams of estragole per kilogram of body weight. Similar groups of 10 animals received corn oil without chemical and served as the control group. At the end of the study, tissues from the animals were examined microscopically for lesions.

Results
All male rats receiving 75 mg/kg or more and all female rats receiving 150 mg/kg or more developed hepatocellular hypertrophy of the liver. Three male rats receiving 600 mg/kg developed cancers of the liver within 3 months. Degeneration of the olfactory epithelium of the nose was seen in rats receiving 300 or 600 mg/kg. Atrophy of the gastric glands of the stomach, cytoplasmic alteration of the salivary glands, and pigmentation and regeneration of renal tubules of the kidney were also observed in rats exposed to estragole. Exposed mice also had noncancerous lesions of the liver and nose and, for female mice, of the stomach.

Conclusion
Exposure to estragole for only 3 months caused cancer of the liver in three out of 10 male rats given 600 mg/kg. Other nonneoplastic lesions were observed in the liver, stomach, nose, kidney and salivary gland of male and female rats and in the liver and nose of male and female mice.

ABSTRACT



Estragole

CAS No. 140-67-0

Chemical Formula: $C_{10}H_{12}O$ Molecular Weight: 148.20

IUPAC Name: 1-methoxy-4-prop-2-enylbenzene

Synonyms: 1-methoxy-4-(2-propenyl)-benzene (9CI); 1-allyl-4-methoxybenzene; 3-(p-methoxyphenyl)propene; 4-allylanisole; 4-allyl-1-methoxybenzene; chavicol methyl ether; esdragol; 1-methoxy-4-(2-propenyl)-benzene; *p*-methoxyallylbenzene; 4-methoxy-2′-propenylbenzene; *p*-allyl-methyl chavicol; tarragon

IUPAC International Chemical Identifier: -InChI=1/C10H12O/c1-3-4-9-5-7-10(11-2)8-6-9/h3,5-8H,1,4H2,2H3

Canonical SMILES: c1(ccc(OC)cc1)CC=C

Estragole is a natural organic compound that is used as an additive, flavoring agent, or fragrance in a variety of food, cleaning, and cosmetic products; as an herbal medicine; as an antimicrobial agent against acid-tolerant food microflora; and to produce synthetic anise oil. Estragole was nominated for toxicity testing by the National Institute of Environmental Health Sciences to characterize its toxicity when administered by gavage to F344/N rats and B6C3F1 mice and to determine how similar its effects might be to those of the structurally related compound, methyleugenol. Male and female F344/N rats and B6C3F1 mice were given estragole (greater than 99% pure) in corn oil by gavage for 3 months. Genetic toxicology studies were conducted in *Salmonella typhimurium* and mouse peripheral blood erythrocytes.

Core and special study (rats only) groups of 10 male and 10 female rats and mice were administered 37.5, 75, 150, 300, or 600 mg estragole/kg body weight in corn oil by gavage, 5 days per week. The core study groups were given estragole for 3 months and the special study groups for 30 days.

All core study rats survived the 3-month exposure period. Mean body weights of the 300 and 600 mg/kg groups were 73% to 92%, respectively, of those of the vehicle control groups. A staining pattern on the ventral surface anterior to

the genitalia beginning at week 9 in the 300 and 600 mg/kg groups was attributed to residue of estragole or metabolites in the urine. Alterations in the erythron related to estragole administration occurred in male and female rats; male rats demonstrated a stronger response. The changes in the erythron were characterized as a microcytic, normochromic, nonresponsive anemia. There were decreases in serum iron concentration in the 300 mg/kg females and 600 mg/kg males and females. The average percent saturation of total iron binding capacity was decreased in the 600 mg/kg males and females. Dose-related increases in platelet counts occurred in most of the dosed groups of rats; the effect appeared to be stronger in males. The increase could be consistent with a reactive thrombocytosis. Increases in the serum alanine aminotransferase and sorbitol dehydrogenase activities suggested a hepatocellular effect (increased leakage) and were consistent with the morphological liver changes observed. There were dose-related increases in serum bile salt concentration in most dosed male rats at all time points; females were less affected.

Absolute and relative liver weights were significantly increased in 300 and 600 mg/kg males and in 75 mg/kg or greater females. Relative kidney weights were significantly increased in all dosed groups of male rats and in female rats given 75 mg/kg or greater. Absolute and relative testis weights of 300 and 600 mg/kg males were significantly decreased.

Two 600 mg/kg male rats had multiple cholangiocarcinomas in the liver and a third had an hepatocellular adenoma. All 600 mg/kg males exhibited cholangiofibrosis. All 75 mg/kg or greater males and all 150 mg/kg or greater females had hepatocellular hypertrophy. Incidences of bile duct hyperplasia, oval cell hyperplasia, and chronic periportal inflammation were significantly increased in all dosed groups. Incidences of basophilic and mixed cell foci were significantly increased in 150 mg/kg or greater males and females. Incidences of eosinophilic focus were significantly increased in 300 and 600 mg/kg males and 600 mg/kg females. Incidences of cellular infiltration of the periportal region by histiocytes increased significantly in all dosed groups of males and in 150 mg/kg or greater females.

Incidences of bone marrow hyperplasia were significantly increased in 75, 300, and 600 mg/kg male rats. Incidences of renal tubule papillary mineralization were significantly increased in 300 mg/kg males and females and 600 mg/kg males. Incidences of cortical renal tubule pigmentation were significantly increased in 150 mg/kg or greater males, and the incidence of renal tubule regeneration was significantly increased in 600 mg/kg females. Incidences of degeneration of the olfactory epithelium in the nose were significantly increased in 300 and 600 mg/kg rats. Incidences of hypertrophied chromophobe cells in the pars distalis of the pituitary gland were significantly increased in 300 and 600 mg/kg males. Cytoplasmic alteration of the submandibular salivary gland occurred in all 75 mg/kg or greater rats. Incidences of atrophy of the gastric glands in the stomach were significantly increased in 150 mg/kg or greater rats. Bilateral degeneration of the germinal epithelium in the testes and bilateral hypospermia of the epididymis occurred in all 300 and 600 mg/kg males.

In the special study, serum gastrin concentration and stomach pH were significantly increased in rats exposed to 600 mg/kg for 30 days. Gastric gland atrophy was significantly increased in the stomach of 300 and 600 mg/kg rats. Hepatic 7-pentoxyresorufin-O-deethylase activity was significantly increased in all exposed groups, except 37.5 mg/kg females, and the increases were generally dose related.

In the mouse core study, a 600 mg/kg male died during week 9, and all 600 mg/kg females died during week 1; the female deaths were attributed to liver necrosis caused by estragole exposure. Mean body weights of 300 and 600 mg/kg males and 75 mg/kg or greater females were 79% to 89% those of the vehicle control groups.

Liver weights were generally increased in 75 mg/kg or greater males and in 300 mg/kg females. Relative thymus weights were significantly increased in all dosed groups of female mice.

The incidences of hepatocellular hypertrophy and hepatocellular degeneration were significantly increased in 300 and 600 mg/kg male mice and 150 and 300 mg/kg female mice. Incidences of oval cell hyperplasia were significantly increased in 300 and 600 mg/kg males and in 75 mg/kg or greater females. Liver necrosis occurred in all 600 mg/kg female mice, along with a significant increase in the incidence of diffuse fatty change. In addition, 600 mg/kg females exhibited significant increases in the incidences of degeneration of the gastric glands of the glandular stomach, as well as squamous hyperplasia, mineralization, and ulcer in the forestomach. Degeneration of the olfactory epithelium in the nose occurred in all 300 and 600 mg/kg mice.

Estragole was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 when tested in the presence or absence of exogenous metabolic activation enzymes. No increases in the frequencies of micronucleated normochromatic erythrocytes were observed in peripheral blood samples from male and female mice in the 3-month study.

Under the conditions of these 3-month studies, estragole showed carcinogenic activity based on the occurrence of two cholangiocarcinomas and one hepatocellular adenoma in the liver of three of 10 male F344/N rats in the high dose group. Because rats and mice were exposed for only 3 months, these studies do not assess the full carcinogenic potential of estragole.

Nonneoplastic effects were observed in the liver, glandular stomach, nose, kidney, and salivary gland of male and female rats and in the testes, epididymides, and pituitary gland of male rats. Nonneoplastic effects were also observed in the liver and nose of male and female mice and in the stomach of female mice.

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Toxicity Study Report on estragole February 28, 2008, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On February 28, 2008, the draft report on the toxicity studies of estragole received public review by the National Toxicology Program's Board of Scientific Counselor's Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. D.W. Bristol, NIEHS, introduced the toxicity studies of estragole by describing the occurrence of the chemical in a variety of plants, its uses as a dietary chemical, the structural characteristics and toxicity of the allyl and isoallyl phenylpropenoid analogues, the design of the 3-month studies of estragole, and the observation of a variety of lesions in rats and mice, including neoplasms in the liver of male rats. Dr. D.E. Malarkey, NIEHS, described the cholangiofibrosis and cholangiocarcinomas observed in the rat study. The proposed conclusions were:

> Estragole is carcinogenic to male F344/N rats based on the occurrence of cholangiocarcinomas and an hepatocellular adenoma in the liver. Because rats and mice were exposed for only 3 months, these studies do not assess the full carcinogenic potential of estragole. The combined evidence from the present study and previously published studies of the genetic toxicity, carcinogenicity, and metabolism of estragole and structurally similar 2-phenylpropene analogues suggests that it is highly likely that estragole would be carcinogenic to additional sites in rats and mice after longer exposures.

Dr. Pino, the first principal reviewer, inquired why an apparent difference in white blood cell count was discounted as an effect. He suggested that some nonneoplastic lesions that did not achieve statistical significance also merited mention and that organ weight changes be identified as primary effects or secondary to other effects. He also suggested that the conclusion statement focus on the target organs.

Dr. Crump, the second principal reviewer, felt some qualifier was needed in the conclusion, rather than simply a statement that the chemical was carcinogenic. He also questioned whether an extrapolation to other sites was warranted in the conclusion statement.

Dr. Bradfield, the third principal reviewer, suggested that transcriptional profiling would be useful and asked for a clearer statement of the purpose of the study.

Dr. G.S. Travlos, NIEHS, described a review of blood smear samples from the studies, which revealed that resistance to lysing of the blood cells in the instrumental measures led to an artifact that appeared as an increase in white cells, which was not confirmed by optical examination of the samples.

Dr. Bristol agreed that the descriptions of organ weight changes and nonneoplastic lesions would be expanded. He said the conclusion statement was consistent with that used in other short-term studies in which neoplasms had been observed. He noted that observing three tumors in a group of only 10 animals after short-term exposure was highly unusual, and that estragole had already been judged to be a carcinogen by the California Environmental Protection Agency.

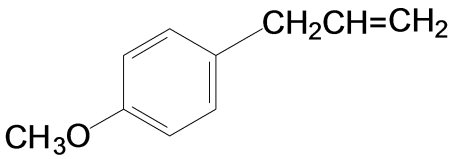
Dr. Mirsalis said that he thought the conclusion statement should be limited to the lesions observed and not predict results of longer term studies. He also noted that the Salmonella and micronucleus genetic toxicity tests were not good predictors for liver carcinogens.

Dr. Pino suggested that the third sentence of the conclusion, regarding prediction of long-term effects, be deleted. Dr. Crump suggested that the first sentence specify the numbers of animals with tumors. He also suggested that the second sentence indicate that the doses used in the short-term study were higher than might be used in a long-term study. Dr. Mirsalis also suggested that the statement about prediction of long-term effects was inappropriate. Dr. Crump felt that such statements might be appropriate for the discussion section.

During a break, a revised conclusion was formulated to incorporate the above suggestions. Dr. Mirsalis moved, and Dr. Soper seconded, that the revised statement be accepted. Drs. M.J. Hooth and J.R. Bucher, NIEHS, said that statements regarding maximum tolerated doses were not applicable for short-term studies such as this. Dr. Mirsalis withdrew the motion. Dr. Pino moved, and Dr. Mirsalis seconded, that the conclusions be accepted with the statement regarding maximum tolerated doses being deleted and nonneoplastic lesions observed be included. The motion was passed unanimously with eight votes. The final statement read:

> Under the conditions of these 3-month studies, estragole showed carcinogenic activity based on the occurrence of two cholangiocarcinomas and one hepatocellular adenoma in the liver of three of 10 male F344/N rats in the high dose group. Because rats and mice were exposed for only 3 months, these studies do not assess the full carcinogenic potential of estragole.
>
> Nonneoplastic effects were observed in the liver, glandular stomach, nose, kidney, and salivary gland of male and female rats and in the testes, epididymides, and pituitary gland of male rats. Nonneoplastic effects were also observed in the liver and nose of male and female mice and in the stomach of female mice.

INTRODUCTION



Estragole

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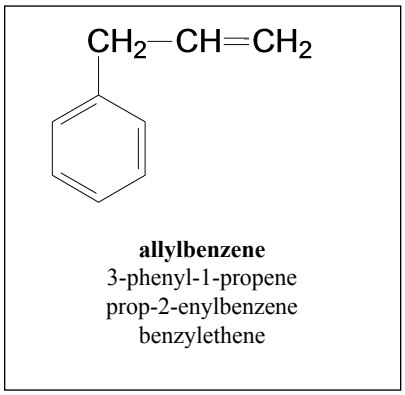
IUPAC International Chemical Identifier: -InChI=1/C10H12O/c1-3-4-9-5-7-10(11-2)8-6-9/h3,5-8H,1,4H2,2H3

Canonical SMILES: c1(ccc(OC)cc1)CC=C

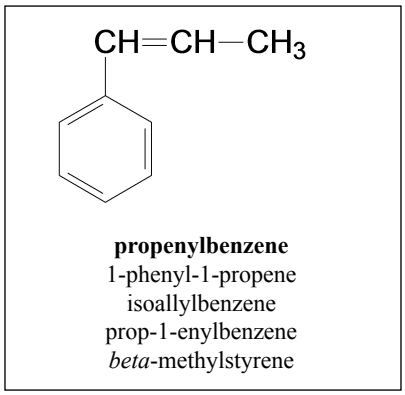
CHEMICAL AND PHYSICAL PROPERTIES

Estragole belongs to a group of volatile plant-derived phenylpropenoid compounds that are biosynthesized from phenylalanine (Koeduka *et al.*, 2008; Pichersky et al, 2006). In pure form it is a colorless liquid with an odor similar to anise (Merck, 1989). It has a molecular weight of 148.20, a boiling point of 216° C, a specific gravity of 0.9645 at 21° C, a refractive index of 1.5230 at 17.5° C, and it is soluble in ethanol and chloroform.

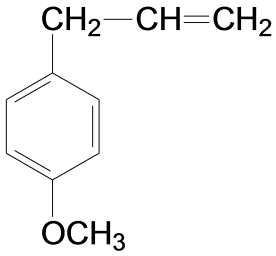
Structurally, phenylpropenoid compounds all have a phenyl group that is attached to an olefinic propenyl group (Figure 1). They are alike in that the phenyl group bears one or more hydroxy, methoxy, or methylenedioxy substituents and is attached to a propene group. The structural feature that partitions them into sets of functionally different analogues, the allylic and propenylic benzenes, is the position that the phenyl ring occupies on the straight-chain, 3-carbon propenyl group. In allylbenzene compounds like estragole, eugenol, methyleugenol, safrole, and myristicin (Figure 1), the phenyl ring [Ph] is attached to the saturated methylene carbon of the propenyl group [Ph-CH_2-CH=CH2]; alternatively this structure can be represented as a benzyl group [Ph-CH_2-] attached to ethene [-CH=CH_2]. In contrast, in propenylbenzene compounds like anethole, isoeugenol, isosafrole, and asarone (Figure 1), the phenyl ring is attached to the terminal methylene carbon of the propenyl double bond [Ph-CH=CH-CH_3]. In propenylbenzenes, the phenyl and propenyl groups form a conjugated system, like that in beta-methylstyrene. Propenylbenzenes are sometimes referred to as isoallylbenzenes.



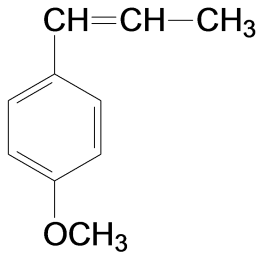
allylbenzene
3-phenyl-1-propene
prop-2-enylbenzene
benzylethene



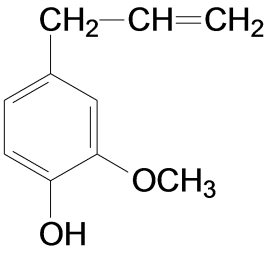
propenylbenzene
1-phenyl-1-propene
isoallylbenzene
prop-1-enylbenzene
beta-methylstyrene



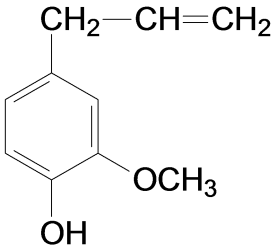
estragole



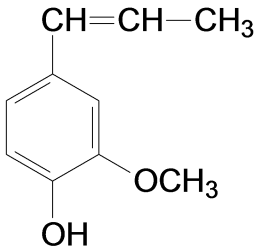
anethole



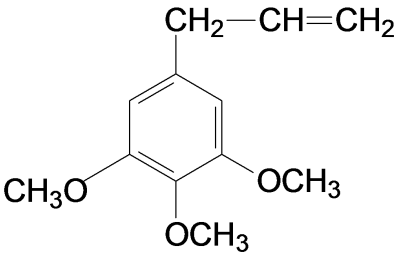
methyleugenol



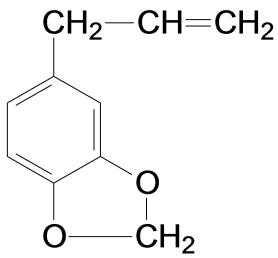
eugenol



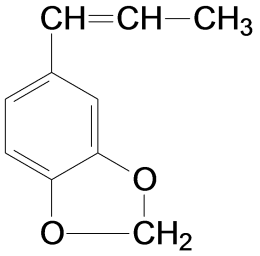
isoeugenol



elemycin
3,4,5-trimethoxyallylbenzene



safrole



isosafrole

Figure 1
Phenylpropenoid Compounds Related to Estragole

PRODUCTION, USE, AND HUMAN EXPOSURE

Estragole occurs naturally in a variety of traditional foods and spices such as basil, anise, fennel, bay leaves, and tarragon. Synthetic estragole is prepared from 4-allylphenol (chavicol) by heating with methyl iodide in methanolic potassium hydroxide or from allyl bromide and *p*-methoxyphenylmagnesium bromide in ether (*Fenaroli's*, 2005). In 1981, approximately 19,980 pounds of estragole were produced in the United States and 17,370 pounds were imported (HSDB, 1998). Estragole is listed in the 1998 High Production Volume Chemical List posted by the United States Environmental Protection Agency (2004), with an estimated annual production volume of 2.8 to 3.8 million pounds. Concentrations of 10 to 45 µg estragole/L have been reported in kraft paper mill wastewaters in Georgia (Keith, 1976), but environmental persistence of estragole is expected to be low (Atkinson, 1990; cited by Guenther *et al.*, 1994).

Human exposure to estragole is universal through its use as an additive, flavoring agent, and fragrance in a variety of foods, cleaning agents, and cosmetic products, as well as from the consumption of the herbs tarragon, basil, and fennel (Drinkwater *et al.*, 1976). It is also used as an antimicrobial agent against acid-tolerant food microflora (Lachowicz *et al.*, 1998; Wan *et al.*, 1998) and to produce synthetic anise oil (Mookherjee and Wilson, 1994).

The essential oil distilled from flowering aerial parts of *Tagetes lucida* Cav. contains 95% to 97% estragole (Cicció, 2004). This aromatic herb occurs naturally from Mexico to Honduras, where it is sold as an infusion, tincture, or elixir and used medicinally to treat an array of common maladies. In the southern United States, this plant is used as a food spice.

The National Institute for Occupational Safety and Health (1990) estimated that approximately 9,128 workers in 668 facilities were potentially exposed to estragole annually. In the general population, the Flavor and Extract Manufacturers Association (FEMA) Expert Panel's estimate for mean daily per capita intake of estragole from all sources is approximately 10 µg/kg body weight per day, with approximately 90% derived from consumption of basil, fennel, tarragon, anise, and their essential oils (Smith *et al.*, 2002). With its higher estragole content, consumption of *Tagetes lucida* (Cicció, 2004) would increase exposures by approximately 50%. The European Union's Scientific Committee on Food (SCF, 2001) estimated the mean daily human intake of estragole to be somewhat higher, at 4.3 mg for a 60 kg person or 70 µg/kg body weight.

REGULATORY STATUS

Estragole is regulated under two statutes by the FDA in the United States. Uses of synthetic estragole as a flavoring substance in baked goods, nonalcoholic beverages, condiments, and hard and soft candy are limited to the minimum quantity needed to produce their intended effect, or to concentrations less than or equal to 50 ppm (21 CFR,

§ 172.515; Hall and Oser, 1965). Uses of essential oils, oleoresins (solvent free), and natural extractives (including distillates) containing estragole (esdragol, esdragon, tarragon) from *Artemisia dracunculus* are classified as Generally Recognized as Safe (GRAS) for their intended use, within the meaning of Section 409 of the Federal Food, Drug, and Cosmetic Act (21 CFR, § 182.20; Hall and Oser, 1965). The FEMA Expert Panel reassessed the safety of estragole in 1979 and 2001 and reaffirmed its GRAS status (Smith *et al.*, 2002).

The regulation of estragole exposure has taken a different path in Europe. In 1981, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) decided not to allocate an acceptable daily intake (ADI), because studies had shown estragole and its metabolites to be mutagenic in bacterial systems and to produce hepatomas in mice (WHO, 1981). The JECFA indicated long-term studies that evaluated the full carcinogenic potential of estragole were needed before an ADI could be established.

In 2000, the Council of Europe, Committee of Experts on Flavouring Substances (CEFS) concluded that estragole is a naturally occurring genotoxic carcinogen in experimental animals after chronic exposure and after few repeated doses and that men dosed with 1 μg/kg, which corresponds to average human exposure, metabolize it to the putative proximate carcinogen 1-hydroxyestragole (Council of Europe, 2000). CEFS recommended an exposure limit of 0.05 mg/kg (detection limit) until needed chronic carcinogenicity studies of estragole in rats and mice of both sexes could be conducted.

A review by the European Union's SCF (2001) concluded that estragole is genotoxic and carcinogenic. Because no safe exposure limit could be established, the SCF recommended that exposure be reduced and use levels restricted. Subsequently, the CEFS classified estragole as a Type I active principle and recommended amounts in food be reduced as far as possible (Council of the European Communities, 2002).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Animals

Estragole is rapidly absorbed, metabolized, and excreted by rodents following either oral or intraperitoneal exposure (Solheim and Scheline, 1973; Drinkwater *et al.*, 1976; Zangouras *et al.*, 1981; Anthony *et al.*, 1987). In these studies, estragole metabolism proceeded through three primary pathways consisting of O-demethylation, 2,3-epoxidation, and 1-hydroxylation. A fourth primary pathway that oxidizes the allyl group to carboxylic acids in mammals was described by Scheline (1991). Formation of the quinone-methide metabolite from O-demethylated estragole, similar to that formed by oxidation of isoeugenol or eugenol (Thompson *et al.*, 1998; NTP, 2010), represents a fifth possible pathway (Figure 2). In general, quinone methides are strongly electrophilic (Meier *et al.*, 2005) and, like 1-hydroxyestragole, may form adducts with cellular nucleophiles such as proteins, RNA, DNA, glutathione, or

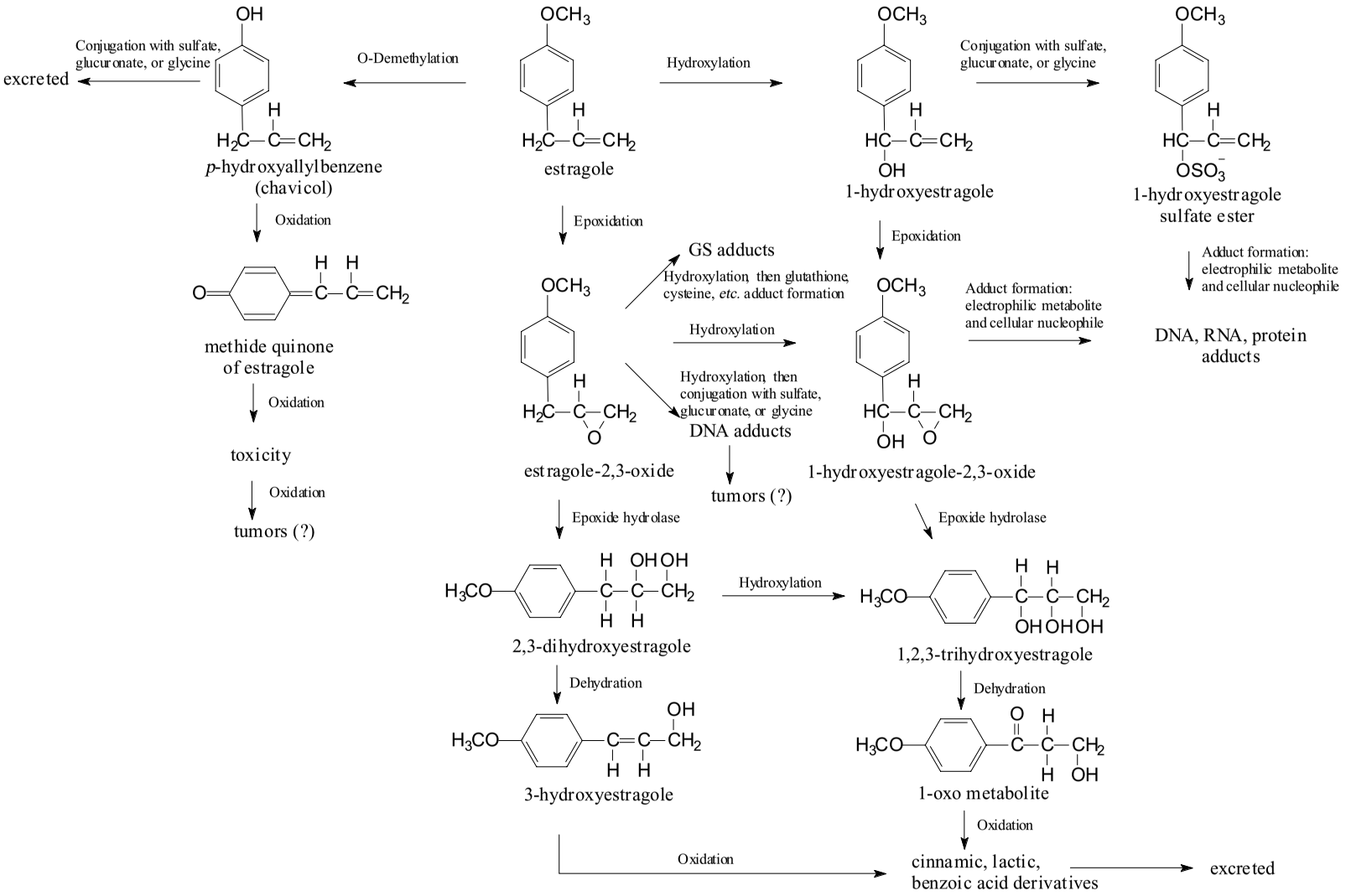


Figure 2
Metabolism of estragole in rats, mice, and humans

cysteine. Figure 2 (adapted from Scheline, 1991; Wakazono *et al.*, 1998 and Rietjens *et al.*, 2005) presents the metabolism of estragole in rats, mice, and humans as several competing pathways whose precise balance is uncertain.

Approximately 23% of a single dose of 274 mg estragole/kg body weight, administered by intraperitoneal injection to 21-day-old and to adult male CD-1 mice, was excreted within 24 hours in urine as conjugated 1-hydroxyestragole (Drinkwater *et al.*, 1976). Zangouras *et al.* (1981) found that *O*-demethylation and 1-hydroxylation are primary pathways in the metabolism of estragole by intraperitoneal-dosed female Wistar rats and male CD-1 mice, with a proportional decrease in *O*-demethylation, apparently due to saturation and an increase in 1-hydroxylation as the dose increased 20,000-fold from 0.05 to 1,000 mg/kg. In female Wistar rats, as the dose increased, excretion of exhaled CO_2 fell from a mean of 34% of the dose to 20%, while urinary excretion rose from a mean of 26% to 53%. The amount of 1-hydroxyestragole in the urine increased from 0.9% of the dose to 8.0% as the dose increased. In male CD-1 mice, as the dose increased, excretion of exhaled CO_2 fell from a mean of 38% to 22%, while urinary excretion rose from a mean of 29% to 50%. The amount of 1-hydroxyestragole in the urine increased from 1.3% to 9.5% as the dose increased. Anthony *et al.* (1987) exposed male CD-1 mice by intraperitoneal injection and female Wistar albino rats by gavage to doses of [methoxy-^{14}C]estragole ranging from 0.05 to 1,000 mg/kg and collected air, urine (50 mg/kg groups), and fecal samples. These investigators also found that the relative amounts of metabolites that contained the radiolabel initially associated with the methoxy group were dose related at higher doses in the 20,000-fold range studied. Rats and mice that received the mid-dose of 50 mg/kg excreted an average of 85% of the radiolabel, 45% as CO_2, 39% in urine, and 1% in feces. In mice, elimination of ^{14}C was essentially complete within 24 hours. At doses up to 50 mg/kg, *O*-demethylation predominated, and as the doses increased 20-fold to 1,000 mg/kg, the relative amount of metabolites in urine increased by approximately 15%. Hydrolysis of 50 mg/kg urine samples at pH 1.0 led to identification of five [methoxy-^{14}C]-containing metabolites: 4-methoxyhippuric acid (6.7% of the dose for mice; 8.2% for rats), 1-hydroxyestragole (mice, 5.2%; rats, 5.4%), 4-methoxyphenylacetylglycine (mice, 3.3%; rats, 1.2%), 4-methoxyphenyllactic acid (mice, 3.0%; rats, 4.5%), and 4-methoxycinnamyl alcohol (mice, 1.5%; rats, 2.9%). The excretion of 2,3-dihydroxy-4-propylanisole, as well as other specific metabolites in the urine, indicated that a portion of the dose was metabolized to estragole 2,3-epoxide in both rats and mice. The recovery of radioactivity from rats and mice exposed to the lowest dose, 0.05 mg/kg, totaled 98% and 82%, respectively.

Safrole and methyleugenol, structural analogues of estragole, are carcinogenic in both sexes of rats and mice (Long *et al.*, 1963; Hagan *et al.*, 1965, 1967; IARC 1976; Johnson *et al*, 2000; NTP, 2000). Like estragole, these allylbenzenes are converted to 1-hydroxy and 2,3-epoxy metabolites (Borchert *et al.*, 1973; Delaforge *et al.*, 1980; Burkey *et al.*, 2000). Evidence indicates that conjugation of 1-hydroxy metabolites with sulfate forms electrophilic 1-sulfate esters, which in turn form adducts with cellular nucleophiles such as DNA, RNA, and proteins, leading to

hepatic carcinogenicity (Boberg *et al.*, 1983; Miller *et al.*, 1983; Miller, 1994; Wakazono *et al*, 1998; Smith *et al.*, 2002; Rietjens *et al.*, 2005). The 2,3-oxide and 1-hydroxy-2,3-oxide metabolites of allylbenzenes also react with nucleophiles to form adducts *in vitro* and *in vivo*; however, some speculate that these oxides are deactivated rapidly *in vivo* by epoxide hydrolase and glutathione-*S*-transferases before they accumulate to toxic levels (Luo and Guenthner, 1996; Guenthner and Luo, 2001). While the 2,3-epoxides of safrole and estragole are genotoxic, they had little or no carcinogenic activity in the liver of rodents (Miller *et al.*, 1983).

Humans

Humans, like rodents, produce 1-hydroxyestragole, even when exposed to a very low amount of estragole. Two male volunteers weighing 93 and 95 kg ingested 100 µg of [methoxy-^{14}C]estragole (Sangster *et al.*, 1987). This one-time, 0.001 mg/kg dose corresponds to the average exposure levels of humans (CEFS, 2000). After 8 hours, 12% had been exhaled as $^{14}CO_2$. Urinary metabolites accounted for 34%, 50%, and 58% of the total dose after 8, 24, and 48 hours, respectively. No unchanged estragole was detected in any samples collected, and no radioactivity was excreted in feces. Larger exposures may result from medicinal uses of essential oils that contain up to 97% estragole (Cicció, 2004). The lowest dose used in similar metabolism studies conducted in rodents was 0.05 mg/kg (Solheim and Scheline, 1973; Zangouras *et al.*, 1981; Anthony *et al.*, 1987). Adjustment of urine pH to 5.0 and hydrolysis with β-glucuronidase released 4% of the dose, including 0.3% as 1-hydroxyestragole, showing that humans convert estragole to its putative proximate carcinogen (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987) even when exposed to levels ordinarily ingested with food. Hydrolysis of urine at pH 1.0 released four other metabolites, identified as 4-methoxyhippuric acid (12%), 4-methoxyphenyllactic acid (4%), 4-methoxycinnamoylglycine (0.8%), and 4-methoxyphenylacetic acid (0.5%). The authors attributed the relatively low 70% recovery of total radiolabel from humans to a poor understanding of the fate of estragole in mammals compared to that of phenylpropenoid analogues. Studies of estragole metabolism in humans, rats, and mice have been reviewed by Scheline (1991) and McDonald (1999).

TOXICITY

Animals

Estragole is marginally toxic following acute exposure. Reported oral LD50 values are 1.2 and 1.8 g/kg for rats and 1.25 g/kg for mice, and the dermal LD50 exceeds 5 g/kg for rabbits (Jenner *et al.*, 1964; Moreno, 1972; both cited by Opdyke, 1976). Rats exposed to four daily oral doses of estragole (605 mg/kg) showed liver discoloration and mottling and blunting of lobe edges, representing minor liver damage (Taylor *et al.*, 1964). Estragole applied full strength was moderately irritating to the intact or abraded skin of rabbits following 24-hour application under occlusion (Moreno, 1972; cited by Opdyke, 1976).

The effect of estragole and estragole-containing essential oils (percent estragole not specified) exposed subcutaneously in peanut oil at a volume of 0.5 mL was evaluated in partially hepatectomized male and female Charles River rats (Gershebin, 1977). Estragole (50 mg/rat per day), tarragon oil, fennel oil, and anise oil significantly increased liver regeneration in male and female rats that were exposed for 7 days following partial hepatectomy and then sacrificed after 10 days. In a further experiment, tarragon oil had no effect on liver regeneration when male and female rats were exposed to 0.50% in the diet for 10 days after partial hepatectomy. Estragon oil, when applied full strength to intact or abraded rabbit skin for 24 hours under occlusion, was irritating (WHO, 1979). It was also irritating when applied undiluted to the backs of hairless mice.

Estragole caused contractions of isolated frog muscles followed by relaxation (Zutshi and Bhagwat, 1977). This effect was reduced in potassium-depolarized muscles or in the presence of quinidine or manganese. Estragole appears to cause these effects by producing a persistent depolarization of the muscle membrane.

Estragole was found to be strongly active in prolonging hexobarbital narcosis and zoxazolamine paralysis in mice injected intraperitoneally with doses from 2.5 to 640 mg/kg (Fujii *et al.*, 1970). This early work concluded that estragole inhibits hepatic microsomal enzyme functions.

Humans

No reports of adverse health effects related to human exposure to estragole were found in the literature. Estragole is used for many purposes in the practice of herbal and folk medicine (Cicció, 2004). Estragon oil, applied as a 4% solution in petrolatum to the skin of 25 human subjects in a 48-hour closed-patch test, produced no irritation (Kligman, 1966).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

No reproductive or developmental toxicity studies of estragole in experimental animals or humans were found in a review of the literature.

CARCINOGENICITY

Animals

Estragole shares structural features with allylbenzene analogues, such as safrole, methyleugenol, and eugenol (Figure 1). The carcinogenicity of these compounds, their known metabolites, and potentially active derivatives has been characterized in comprehensive sets of rodent bioassays in multiple mouse strains. The series of studies with

estragole demonstrate that it is hepatocarcinogenic in CD-1 and B6C3F1 mice following oral, intraperitoneal, or subcutaneous exposure. Weanling male mice were found to be more susceptible than females. In addition, the 1-hydroxy metabolite of estragole was shown to be a stronger hepatocarcinogen than the parent compound (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987).

Estragole induced hepatomas in male CD-1 mice, whether exposed by gavage or in the diet. Male and female preweanling CD-1 mice were exposed to 2.5 mmol estragole/kg body weight (370 mg/kg) in trioctanoin by gavage twice weekly for 5 weeks (Miller *et al.*, 1983). Within 11 to 14 months, a significant increase of hepatomas was observed in male (73%), but not female (9%), mice compared with controls (24% and 9%, respectively). In contrast, 8-week old female CD-1 mice fed diets containing 0.23% or 0.46% estragole or 0.25% 1-hydroxyestragole for 12 months developed significant, dose-related increases in hepatomas (Miller *et al.*, 1983). At 20 months, 56%, 71%, and 56% of the mice, respectively, developed liver tumors compared to 0% of control animals.

Hepatomas were increased in preweanling CD-1 male mice that received four intraperitoneal injections of estragole (totaling 9.45 µmol) on postpartum days 1, 8, 15, and 22; incidences were not increased with estragole-2,3-oxide (Miller et al., 1983). Likewise, hepatomas were increased in preweanling B6C3F1 mice that received single or four intraperitoneal injections of estragole, 1-hydroxyestragole, or 1-hydroxy-2,3-dehydroestragole (Miller *et al.*, 1983; Wiseman *et al.*, 1987). Wiseman *et al.* (1987) identified an age effect on liver tumor induction in preweanling B6C3F1 male mice by injecting the same dose of 1-hydroxyestragole per gram of body weight on day 1 or day 12 of age. Approximately twice as many hepatomas per liver were observed in mice injected on day 12 of age compared to day 1 of age. Hepatomas were also significantly increased in preweanling CeH/HeJ male mice injected intraperitoneally with 1-hydroxyestragole, but not in female CeH/HeJ mice or in male or female C57BL/6J mice. Estragole, 1-hydroxyestragole, or estragole-2,3-oxide did not induce lung adenoma at 8 months in female A/J mice injected twice weekly for 12 weeks (Wiseman *et al.*, 1987); however, significant increases were induced by the estragole metabolite, 1-hydroxyestragole-2,3-oxide.

Estragole and 1-hydroxyestragole also induced hepatomas in mice when exposed by subcutaneous injection. Increased incidences of hepatoma were observed in groups of preweanling male CD-1 mice exposed to four subcutaneous injections of estragole or 1-hydroxyestragole in trioctanoin on days 1, 8, 15, and 22 (Drinkwater *et al.*, 1976). At 15 months, the incidences of hepatocellular carcinomas in animals receiving a total dose of 4.4 or 5.2 µmol (650 or 770 µg, respectively) estragole were 23% and 39%, respectively, compared to 12% of vehicle control mice. Hepatomas developed in 70% of male CD-1 mice that received a total dose of 4.4 µmol 1-hydroxyestragole.

Sulfate esters of the 1-hydroxy metabolite are implicated as the major ultimate electrophilic and carcinogenic metabolites of 1-hydroxyestragole and of other allylbenzene analogues (Boberg *et al.*, 1983; Wiseman *et al.*, 1987; Miller, 1994). For example, the formation of an average of 6.6 liver tumors per animal in 95% of 12-day old B6C3F1 male mice that received a single intraperitoneal dose of 0.75 mmol (110 mg) of estragole/kg body weight was completely inhibited by pretreatment with pentachlorophenol, a potent sulfotransferase inhibitor (Wiseman *et al.*, 1987). Studies using brachymorphic mice deficient in sulfotransferase also strongly inhibited liver tumor induction by estragole and safrole (Boberg *et al.*, 1983).

Estragole-2,3-oxide and 1-hydroxyestragole-2,3-oxide induced benign skin tumors in 8-week-old female CD-1 mice exposed topically 4 days/week for 6 weeks followed by repeated application of croton oil (Miller *et al.*, 1983).

Safrole, fed to male and female Osborne-Mendel rats at up to 5,000 ppm in feed for 2 years, induced significant increases in hepatocellular carcinoma, hepatocellular adenoma, hepatocholangiocarcinoma, and hepatocholangioma (Long *et al.*, 1963). Mice exposed to 464 mg/kg safrole by gavage from postnatal days 7 to 28 and then to 1,112 ppm safrole in feed developed liver neoplasms (Innes *et al.*, 1969). Male B6C3F1 mice nursed by mothers exposed to safrole also developed liver tumors (Vesselinovitch *et al.*, 1979).

Although the carcinogenic activity of estragole has not been characterized in rats, one study evaluated the effects of estragole and safrole metabolites in male Fisher rats. Groups of 20 received either 1-hydroxy-, 2,3-epoxy-, or 1-hydroxy-2,3-epoxy-metabolites of estragole or safrole by subcutaneous injection twice weekly for 20 weeks (total dose of 2 mmol/rat). At 2 years, only 1-hydroxysafrole induced a significant increase ($P < 0.001$) in tumors. Of 11 male Fisher rats with hepatic carcinomas, seven had hepatocellular carcinomas, one had cholangiocarcinoma, and three had mucinous adenocarcinomas of biliary origin; a 12th rat had cholangioma (Wiseman *et al.*, 1987).

The NTP conducted bioassays for carcinogenesis with eugenol, methyleugenol, and most recently isoeugenol. Eugenol, fed at 0, 3,000 or 6,000 ppm in feed for 2 years, induced weak increases of hepatic adenoma and carcinoma in male and female B6C3F1 mice, but no tumors in F344/N rats (NTP, 1983). In contrast, exposure to methyleugenol by gavage produced liver tumors in rats after relatively short intervals. Exposure for 14 weeks to 1,000 mg/kg resulted in a diagnosis of hepatocellular adenoma in one male rat (Abdo *et al.*, 2001). This observation prompted the inclusion of interim evaluations in the subsequent chronic toxicity and carcinogenicity study. After just 12 months of exposure to 300 mg/kg of methyleugenol, hepatocellular adenoma was diagnosed in four male rats (including two with multiple hepatocellular adenomas), another had an hepatocholangiocarcinoma, and hepatocellular carcinoma was observed in one female rat; however, no tumors were observed in mice (Johnson *et al.*, 2000; NTP, 2000). Exposure to methyleugenol by gavage for 2 years induced benign and malignant liver neoplasms in both sexes of

F344/N rats and B6C3F1 mice, neuroendocrine tumors of the glandular stomach in rats and male mice, as well as kidney neoplasms, mesotheliomas, mammary gland fibroadenomas, and subcutaneous fibromas and fibrosarcomas in male rats (NTP, 2000). Methyleugenol also induced nonneoplastic fundic mucosal atrophy and neuroendocrine hyperplasia of the glandular stomach (Johnson *et al.*, 2000; NTP, 2000). Furthermore, it decreased gastric secretion (hypochlorhydria), increased intragastric pH, and increased serum gastrin (hypergastrinemia). Thake *et al.* (1995) attributed these effects on the glandular stomach to a cytotoxic loss of parietal cells that inhibits gastric acid secretion and, when prolonged, induces enterochromaffin-like cell tumors. Thus, increased pH and gastrin secretion may stimulate hyperplasia of neuroendocrine cells, which may ultimately lead to tumor formation. In light of the methyleugenol study results, intragastric pH and serum gastrin were measured during the subchronic study of estragole in rats.

Humans

No epidemiology studies or case reports associating estragole exposure with cancer risk in humans were found in the literature. Methyleugenol is listed in the NTP Report on Carcinogens as reasonably anticipated to be a human carcinogen based on sufficient evidence of its carcinogenicity in experimental animals (NTP, 2004a). Safrole is classified by the International Agency for Research on Cancer (1976) as possibly carcinogenic to humans (Group 2B) and is listed as reasonably anticipated to be a human carcinogen in the NTP Report on Carcinogens (NTP, 2004b).

GENETIC TOXICITY

Estragole was not mutagenic in any of several strains of *Salmonella typhimurium,* with or without hamster or rat liver S9 metabolic activation enzymes (Drinkwater *et al.*, 1976; Swanson *et al.*, 1979; Sekizawa and Shibamoto, 1982; Zeiger *et al.*, 1987). Neither did it induce DNA damage in the *Bacillus subtilis Rec* assay (Sekizawa and Shibamoto, 1982) or chromosomal aberrations *in vitro* in hamster V79 cells, with or without metabolic activation provided by rat liver S9 or rat hepatocytes in co-culture (Müller *et al.*, 1994). However, use of rat liver extract S13 to activate estragole resulted in mutagenic responses in *S. typhimurium* strain TA100 (Drinkwater *et al.*, 1976; Swanson *et al.*, 1979), which mutates via base substitution.

Estragole induced dose-related increases in unscheduled DNA synthesis (UDS; indicative of DNA damage) in rat hepatocytes treated *in vitro* (10^{-6} to 10^{-3} M) or *in vivo* (0.5 to 2.0 g/kg) (Müller *et al.*, 1994). A comparative study of estragole, methyleugenol, and safrole showed that dose-related increases in UDS in cultured F344 rat hepatocytes were induced by each of these allylbenzenes and that levels of UDS were enhanced, following exposure to the 1-hydroxy metabolites of these compounds (Chan and Caldwell, 1992). DNA adducts were increased in livers of adult CD-1 female mice or newborn B6C3F1 male mice following intraperitoneal injections of estragole (Phillips *et al.*, 1984; Randerath *et al.*, 1984). Although the same types of nucleoside adducts were identified in the liver of

both the adult and newborn mice, they persisted longer in newborn B6C3F1 male mice. The greater persistence of estragole-induced DNA adducts in the liver of newborn male mice was suggested to be directly related to the observed greater sensitivity of newborn versus adult male mice to tumor induction by estragole (Phillips *et al.*, 1984). Comparison of DNA-liver adducts formed in newborn and adult mice exposed to safrole or methyleugenol revealed a spectrum of adducts similar to those formed by estragole (Phillips *et al.*, 1984; Randerath *et al.*, 1984). In further experiments, preexposure of female CD-1 mice to pentachlorophenol, a known inhibitor of sulfotransferases, was shown to inhibit safrole-induced adduct formation in liver DNA, providing evidence that the activation of safrole (as well as estragole and methyleugenol) to the ultimate electrophilic genotoxic and carcinogenic metabolite proceeds through generation of 1-hydroxy and 1-sulphoxy derivatives (Randerath *et al.*, 1984). Analysis of results from comparative studies of DNA adduct formation and tumor induction in mice (Phillips, *et al.*, 1984; Wiseman *et al.*, 1985) indicates that allylbenzene compounds bearing methoxy or methylenedioxy substituents at the 3′- and/or 4′-phenyl positions (i.e., estragole, safrole, methyleugenol) have relatively high *in vivo* DNA binding capability, and perhaps enhanced carcinogenic potential, compared to the more highly substituted allylbenzenes, elemicin (Figure 1), dill apiole, parsley apiole, or myristicin.

A number of *in vitro* and *in vivo* studies have been performed to identify the metabolites of estragole that pose a hazard for genotoxicity. They indicate that epoxidation generates highly reactive 2,3-oxide metabolites that can react efficiently with macromolecules; however, *in vitro* studies indicate that these metabolites are rapidly rendered inactive by a number of detoxification enzymes (epoxide hydrolases) before they can accumulate enough to induce genotoxicity via DNA adduct formation (Guenthner and Luo, 2001). These 2,3-oxides are mutagenic to *S. typhimurium* strain TA100 in the absence of exogenous metabolic activation; addition of S13 liver homogenate eliminated the mutagenic activity, implicating sulfate ester formation with activity (Swanson *et al.*, 1979). The primary metabolite believed to be responsible for the genotoxicity and carcinogenicity of estragole is 1-hydroxyestragole. It was demonstrated to be weakly mutagenic in *S. typhimurium* strain TA100 in the absence of exogenous metabolic activation, and addition of S13 liver homogenate enhanced the mutagenic response. The spectra of liver-DNA adducts in mice exposed to 1-hydroxyestragole and the putative proximate carcinogen of safrole, 1-hydroxysafrole, were similar (Phillips *et al.*, 1981; Wiseman *et al.*, 1985).

STUDY RATIONALE

Estragole is a high production volume chemical that is added to foods as a flavoring substance and is used as a fragrance in cosmetics and cleaning agents. As a result, there is high potential for human exposure. Published studies indicate that estragole, like the allylbenzene analogues safrole and methyleugenol, is genotoxic and carcinogenic. Specifically, estragole is hepatocarcinogenic in preweanling mice following oral, intraperitoneal, or subcutaneous exposure, is genotoxic in *S. typhimurium* TA100 using rat liver extract S13, induces unscheduled DNA synthesis, and

forms DNA adducts. Estragole is metabolized in rodents and humans to 1-hydroxyestragole and to the corresponding sulfate ester, which is the putative ultimate carcinogen formed from allylbenzene analogues. Based on these similarities, the current study was conducted to better characterize the toxicity and potential carcinogenicity of estragole in F344/N rats and B6C3F1 mice. To facilitate direct comparison with NTP studies of methyleugenol and isoeugenol, gavage was chosen as the route of exposure, comparable doses were selected, and similar special-study endpoints were included.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF ESTRAGOLE

Estragole was obtained from Penta International Corporation (Livingston, NJ) in one lot (57533). The analytical chemistry laboratory, Battelle Columbus Operations (Chemistry Support Services, Columbus, OH), performed vacuum distillation on lot 57533, and the distilled product was assigned lot number 021401 and was used during the 3-month studies. Identity and purity analyses were conducted by the analytical chemistry laboratory and the study laboratory, Battelle Columbus Operations (Columbus, OH) (Appendix E). Karl Fischer titration and elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). Reports on analyses performed in support of the estragole studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a clear, colorless liquid, was identified as estragole using infrared and proton and carbon-13 nuclear magnetic resonance spectroscopy. The purity of lot 021401 was determined by elemental analysis, gas chromatography (GC), high-performance liquid chromatography (HPLC), and potentiometric titration.

Karl Fischer titration indicated 194 ppm water. Elemental analyses for carbon, hydrogen, and oxygen were in agreement with the theoretical values for estragole. GC by one system indicated one major peak and no impurities greater than 0.1% of the total peak area; purity was determined to be greater than 99%. GC by another system indicated a purity of 99.5% relative to a frozen reference sample of the same lot. HPLC indicated one major peak and no impurities greater than 0.1% of the total peak area. Potentiometric titration indicated the peroxide concentration was below the acceptable limit of 3 mEq/kg. The overall purity of lot 021401 was determined to be greater than 99%.

To ensure stability, the bulk chemical was stored in sealed amber glass bottles under an argon headspace, protected from light, at approximately –20° C. Reanalysis of the bulk chemical was performed twice during the studies using gas chromatography and potentiometric titration to determine peroxide concentrations. No degradation of the bulk chemical was detected, and peroxide concentrations were within acceptable limits.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared three times during the 3-month studies by mixing estragole with corn oil (Table E1). The analytical chemistry laboratory performed homogeneity studies (0.2 and 120 mg/mL dose

formulations), a gavageability study (120 mg/mL dose formulation), and stability studies (0.2 mg/mL dose formulation) using gas chromatography. Homogeneity and gavageability were confirmed, and stability was confirmed for at least 42 days for dose formulations stored in amber glass bottles protected from light at approximately –20°, 5°, or 25° C and for at least 3 hours under simulated animal room conditions.

Periodic analyses of the dose formulations of estragole were conducted by the study laboratory using GC. During the 3-month studies, the dose formulations were analyzed three times; animal room samples of these dose formulations were also analyzed (Table E2). All dose formulations were within 10% of the target concentrations. Corn oil was obtained in two lots (QQ0053 and QN0035) from Spectrum Chemicals and Laboratory Products (Gardena, CA) and was used as the vehicle during the 3-month studies. The study laboratory determined peroxide concentrations three times during the studies using potentiometric titration; all peroxide concentrations were less than the acceptable limit of 3 mEq/kg.

3-MONTH STUDIES

Dose levels were selected based on a review of published literature and methyleugenol studies (NTP, 2000). Male and female F344/N rats and B6C3F1 mice were obtained from Taconic Farms, Inc. (Germantown, NY). On receipt, the rats and mice were 4 to 5 weeks old. Animals were quarantined for 11 to 14 days and were 5 to 7 weeks old on the first day of the studies. Before the studies began, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. Blood was collected from five male and five female sentinel rats and mice at 4 weeks and at 3 months. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b). All results were negative.

Core study groups of 10 male and 10 female rats and mice and special study groups of 10 male and 10 female rats received estragole in corn oil by gavage at doses of 37.5, 75, 150, 300, or 600 mg/kg body weight 5 days per week. The core study groups were given estragole for 3 months and the special study groups for 30 days. Vehicle control animals received the corn oil vehicle alone. Feed and water were available *ad libitum*, except special study rats were fasted for 24 hours prior to blood collection on day 31. Rats and female mice were housed five per cage; male mice were housed individually. All animals were observed twice daily. Clinical findings were recorded initially, weekly, and at necropsy for core study rats and mice. The animals were weighed initially, weekly, and at necropsy. Details of the animal husbandry and study conduct are summarized in Table 1.

Animals were anesthetized with a carbon dioxide and oxygen mixture. Blood was collected from the retroorbital sinus of special study rats on days 4 and 23 and from core study rats and mice at terminal sacrifice for hematology

(rats and mice) and clinical chemistry (rats) analyses. Blood samples for hematology analyses were placed in tubes containing potassium EDTA. Erythrocyte, leukocyte, and platelet counts; hemoglobin concentration; mean cell volume; mean cell hemoglobin; and mean cell hemoglobin concentration were determined using a Cell-Dyn® 3500 hematology analyzer (Abbott Diagnostics, Abbott Park, IL). Blood samples for clinical chemistry analyses were placed in tubes containing separator gel and allowed to clot. After clot retraction occurred, the samples were centrifuged, and the serum chemistry was analyzed using a Hitachi® 911. Table 1 lists the parameters measured.

On day 31, blood was collected from the retroorbital sinus of special study rats. Serum was separated and stored at –70° C until analysis. Serum gastrin levels were determined by radioimmunoassay (American Laboratory Products Company, Windham, NH). Following blood collection on day 31, special study rats were euthanized with carbon dioxide, and the stomach was isolated for pH determination. The duodenum distal to the pylorus was incised; a Model Number PHR-146 pH electrode (Jenco Instruments, Inc., San Diego, CA) was inserted into the stomach, and a Model Number 6250 pH meter (Jenco Instruments, Inc.) was used to measure stomach pH. After recording the pH, the stomach was fixed, embedded, and stained for histopathologic examination.

During the stomach pH measurement, a liver sample was collected from special study rats, weighed, and stored at –70° C for cytochrome P450 determinations. Microsomal suspensions were prepared using the Pearce method (Pearce *et al.*, 1996). The concentration of protein in each suspension was determined using the microtiter plate method of the Coomassie® Plus Protein Assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard. Cytochrome P450 1A1 (CYP1A1)-associated 7-ethoxyresorufin-*O*-deethylase, CYP1A2-associated acetanilide-4-hydroxylase, and CYP2B-associated 7-pentoxyresorufin-*O*-deethylase activities were determined in microsomal protein according to established procedures.

Necropsies were performed on all core study animals. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (eyes were fixed in Davidson's solution for up to 72 hours and then transferred to 10% neutral buffered formalin), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on animals that died early, vehicle control rats and mice, 600 mg/kg rats and mice, and 300 mg/kg female mice. In addition, target tissues were examined microscopically in all groups. Table 1 lists the tissues and organs routinely examined, as well as the target tissues for rats and mice exposed to estragole.

Upon completion of the laboratory pathologist's histopathologic evaluation, the slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives. The slides, individual animal data records, and pathology tables were sent to an independent pathology laboratory where quality assessment was performed. Results were reviewed and evaluated by the NTP Pathology Working Group (PWG); the final diagnoses represent a consensus of the laboratory pathologist(s), reviewing pathologist(s), and the PWG. Details of these review procedures have been described by Maronpot and Boorman (1982) and Boorman *et al.* (1985).

TABLE 1
Materials and Methods in the 3-Month Gavage Studies of Estragole

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

Strain and Species
F344/N rats
B6C3F1 mice

Animal Source
Taconic Farms, Inc. (Germantown, NY)

Time Held Before Studies
Rats: 11 (males) or 12 (females) days
Mice: 13 (females) or 14 (males) days

Average Age When Studies Began
5 to 7 weeks

Date of First Dose
Rats: September 10 (males) or 11 (females), 2001
Mice: September 12 (females) or 13 (males), 2001

Duration of Dosing
5 days/week for 14 weeks

Date of Last Dose
Rats: December 10 (males) or 11 (females), 2001
Mice: December 12 (females) or 13 (males), 2001

Necropsy Dates
Rats: December 11 (males) or 12 (females), 2001
Mice: December 13 (females) or 14 (males), 2001

Average Age at Necropsy
19 to 20 weeks

Size of Study Groups
Rats: 10 males and 10 females (core study)
10 males and 10 females (special study)
Mice: 10 males and 10 females

Method of Distribution
Animals were distributed randomly into groups of approximately equal initial mean body weights.

Animals per Cage
Rats: 5
Mice: 1 (males) or 5 (females)

Method of Animal Identification
Tail tattoo

Diet
NTP-2000 irradiated wafer diet (Zeigler Brothers, Inc., Gardners, PA); available *ad libitum*, except special study rats were fasted for 24 hours before blood collection on day 31; changed weekly

Water
Tap water (Columbus, OH, municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*

Cages
Polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly (male mice) or twice weekly (rats and female mice)

TABLE 1
Materials and Methods in the 3-Month Gavage Studies of Estragole

Bedding
Irradiated Sani-Chips® (P.J. Murphy Forest Products Corporation, Montville, NJ), changed weekly (male mice) or twice weekly (rats and female mice)

Cage Filters
Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH), changed every 2 weeks

Racks
Stainless steel (Lab Products, Inc.), changed every 2 weeks

Animal Room Environment
Temperature: 72° ± 3° F
Relative humidity: 50% ± 15%
Room fluorescent light: 12 hours/day
Room air changes: at least 10/hour

Doses
0, 37.5, 75, 150, 300, or 600 mg/kg in corn oil by gavage (dosing volumes: 5 mL/kg for rats and 10 mL/kg for mice)

Type and Frequency of Observation
Observed twice daily. Clinical findings were recorded initially, weekly, and at necropsy for core study rats and mice. The animals were weighed initially, weekly, and at necropsy.

Method of Sacrifice
Carbon dioxide asphyxiation

Necropsy
Necropsies were performed on all core study animals. Organs weighed were heart, right kidney, liver, lung, right testis, and thymus.

Clinical Pathology
Blood was collected from the retroorbital sinus of special study rats on days 4 and 23 and from core study rats at terminal sacrifice for hematology and clinical chemistry. Blood was collected from the retroorbital sinus of mice at terminal sacrifice for hematology.
Hematology: hematocrit; hemoglobin; erythrocyte, reticulocyte, nucleated erythrocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials
Clinical chemistry: urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, bile acids, iron, unsaturated iron binding capacity, and total iron binding capacity

Histopathology
Complete histopathologic examinations were performed on animals that died early, vehicle control rats and mice, 600 mg/kg rats and mice, and 300 mg/kg female mice. In addition to grossly observed lesions and masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eye, gallbladder (mice), harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung (with mainstem bronchus), lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus. Target tissues were examined in all groups. Nontarget tissues were examined to a no-effect level. Target tissues in rats included the bone marrow (males), epididymis (males), kidney, liver, lung, mesenteric lymph node, nose, pituitary gland (males), salivary gland, glandular stomach, and testis (males); target tissues in mice included the kidney, liver, mandibular and mesenteric lymph nodes, nose, spleen, forestomach, and glandular stomach.

Special Study
On day 31, blood was collected from the retroorbital sinus of special study rats for serum gastrin analysis, the stomach was isolated for pH determination, and liver samples were taken for determinations of hepatic tissue mass, microsomal protein, acetanilide-4-hydroxylase, 7-ethoxyresorufin-*O*-deethylase, and 7-pentoxyresorufin-*O*-deethylase. Stomach was fixed, embedded, and stained for histopathologic evaluation.

STATISTICAL METHODS

Calculation and Analysis of Lesion Incidences

The incidences of lesions are presented in Appendix A as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test (Gart *et al.,* 1979), a procedure based on the overall proportion of affected animals, was used to determine significance and the Cochran-Armitage trend test was used to test significant trends between dosed and vehicle control animals (Armitage, 1971).

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed and vehicle control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology, clinical chemistry, serum gastrin, stomach pH, and cytochrome P450 data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1957) were examined by NTP personnel, and implausible values were eliminated from the analysis.

QUALITY ASSURANCE METHODS

The 3-month studies were conducted in compliance with FDA Good Laboratory Practice Regulations (21 CFR, Part 58). The Quality Assurance Unit of Battelle Columbus Operations performed audits and inspections of protocols, procedures, data, and reports throughout the course of the studies. The completeness and accuracy of factual information presented in this report was assessed by an NTP quality assessment support contractor before publication.

GENETIC TOXICOLOGY

Salmonella typhimurium Mutagenicity Test Protocol

Testing was performed as reported by Zeiger *et al.* (1987). Estragole was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 either in buffer or S9 mix (metabolic activation enzymes and cofactors from

Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of five doses of estragole. The high dose was limited by toxicity. All trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. Although positive calls are typically reserved for increases in mutant colonies that are at least twofold over background, there is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

Mouse Peripheral Blood Micronucleus Test Protocol

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). At terminal sacrifice, peripheral blood samples were obtained from male and female mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes (NCEs) in each of five animals per dose group except 600 mg/kg females, because all animals in this dose group died the first week of the study. In addition, the percentage of polychromatic erythrocytes (PCEs) among the total erythrocyte population in the bone marrow was scored for each dose group as a measure of bone marrow toxicity.

The results were tabulated as the mean of the pooled results for each group plus or minus the standard error of the mean. The frequency of micronucleated cells among NCEs was analyzed by a statistical software package that tested for increasing trend over dose groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dosed group and the control group. In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dosed group is less than or equal to 0.025 divided by the number of dosed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Results of the 3-month study were accepted without repeat tests,

because additional test data could not be obtained. Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects.

Evaluation Protocol

These are the basic guidelines for arriving at an overall result for short-term assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay, and different results were obtained among aliquots and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The results presented in the Abstract of this Toxicity Study Report represent a scientific judgement of the overall evidence for activity of the chemical in an assay.

RESULTS

3-Month Study in Rats

All rats survived to terminal sacrifice; mean body weights and mean body weight gains of the 300 and 600 mg/kg groups were significantly less than those of the vehicle control groups (Table 2; Figure 3). The only clinical finding related to estragole exposure was staining on the ventral surface anterior to the genitalia beginning at week 9 in the 300 mg/kg groups (males, 7/10; females, 3/10) and the 600 mg/kg groups (males, 4/10; females, 9/10).

Table 2
Survival and Body Weights of Rats in the 3-Month Gavage Study of Estragole

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight[c] Relative to Controls (%)
		Initial	Final[c]	Change	
Male					
0	10/10	90 ± 2	327 ± 5	237 ± 5	
37.5	10/10	90 ± 2	333 ± 5	243 ± 3	102
75	10/10	89 ± 2	329 ± 3	240 ± 4	101
150	10/10	89 ± 2	316 ± 4	227 ± 3	97
300	10/10	88 ± 3	278 ± 4**	190 ± 4**	85
600	10/10	89 ± 2	238 ± 3**	149 ± 3**	73
Female					
0	10/10	82 ± 2	193 ± 4	111 ± 4	
37.5	10/10	82 ± 2	193 ± 4	111 ± 3	100
75	10/10	83 ± 2	191 ± 3	108 ± 3	99
150	10/10	83 ± 2	186 ± 2	103 ± 2	97
300	10/10	83 ± 2	177 ± 3**	94 ± 3**	92
600	10/10	83 ± 2	168 ± 3**	85 ± 2**	87

** Significantly different (P≤0.01) from the vehicle control group by Williams' test
[a] Number of animals surviving at 3 months/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Day 85

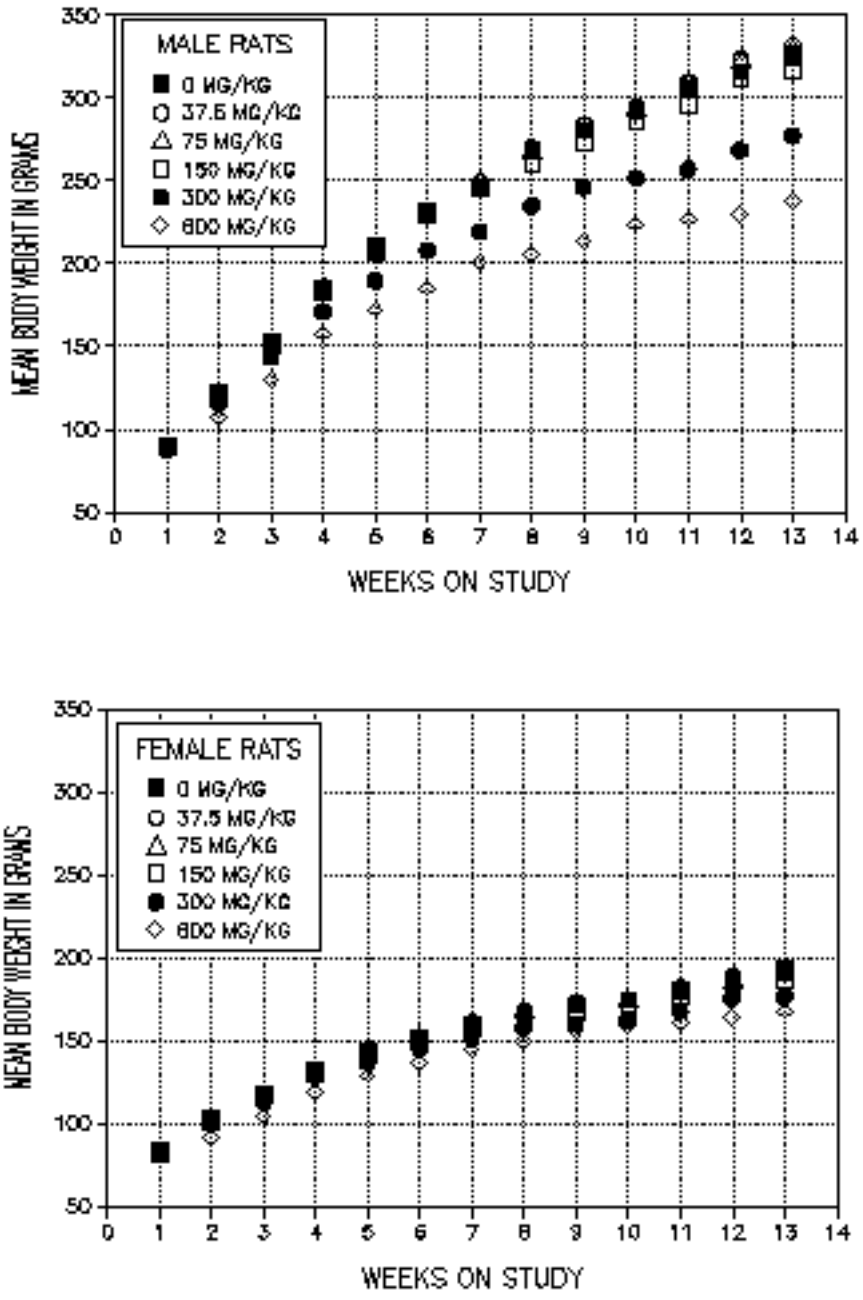


FIGURE 3
Growth Curves for Male and Female Rats Administered Estragole by Gavage for 3 Months

Hematology and clinical chemistry data for rats are presented in Tables 3 and C1. Alterations in the erythron related to estragole occurred in male and female rats; male rats demonstrated a stronger response. By day 23, an anemia, evidenced by decreases in hematocrit and hemoglobin values, occurred in the 600 mg/kg males and females, extending to the 300 mg/kg males at week 14. There were no changes in erythrocyte counts on day 23. But at week 14, erythrocyte counts for the 600 mg/kg groups increased by approximately 10%. Neither on day 23 nor at week 14 was there an indication of an erythropoietic response, evidenced by no change in reticulocyte or nucleated erythrocyte counts. The mean cell volume indicated decreases in mean erythrocyte size throughout the study, consistently affecting the 300 and 600 mg/kg groups; the mean cell volume decrease intensified with time in the 600 mg/kg groups. The mean cell hemoglobin values demonstrated decreases that essentially mirrored the mean cell volume decreases. The mean cell hemoglobin concentration values, however, remained stable throughout the study. Thus, the changes in the erythron were characterized as a microcytic, normochromic, nonresponsive anemia and would be consistent with an ineffective erythropoiesis.

The erythrocyte morphology demonstrated a general increase in erythrocyte central pallor and increased numbers of target cells, microcytes, and erythrocyte fragments (Plate 1). The increase in erythrocyte central pallor would also be consistent with decreased heme or hemoglobin production within developing erythrocytes. The microcytes could also reflect decreased heme or hemoglobin production and would be consistent with the decreased mean cell volume. Target cells are usually a result of hemoglobin redistribution within the erythrocyte and, in humans, have been observed with liver disease, hypochromic anemias, and some hemoglobinopathies. The increased numbers of erythrocyte fragments would suggest increased erythrocyte fragility or erythrocyte trauma; the mechanism in this study is unknown.

On day 23 and at week 14, dose-related increases in platelet counts occurred in most dosed males and females; the effect appeared to be stronger in males. The mechanism of the platelet count increase is unknown, but it could be consistent with a reactive thrombocytosis (increased production) that has been observed with a variety of conditions (e.g., iron deficiency, inflammation). At week 14, an apparent dose-dependent increase in the leukocyte count occurred in dosed males and females; the leukocyte count increase was characterized by increases in lymphocyte and neutrophil counts. While this change could suggest a physiological response, estimated leukocyte counts from the blood smears did not corroborate the increased numbers, suggesting that the instrument-derived leukocyte counts were erroneously elevated; the cause for this elevation is unknown.

TABLE 3
Selected Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
Hematology						
n						
Day 4	10	10	10	10	10	10
Day 23	10	9	10	10	10	10
Week 14	10	10	10	10	9	10
Hematocrit (%)						
Day 4	40.7 ± 0.7	40.2 ± 0.5	40.1 ± 0.4	40.8 ± 0.5	40.0 ± 0.3	41.9 ± 0.6
Day 23	45.5 ± 0.4	44.8 ± 0.5	45.3 ± 0.4	45.7 ± 0.2	44.4 ± 0.3	35.8 ± 1.3**
Week 14	45.1 ± 0.4	43.6 ± 0.3	44.3 ± 0.3	44.6 ± 0.2	42.7 ± 0.4**	33.5 ± 1.0**
Hemoglobin (g/dL)						
Day 4	13.2 ± 0.2	13.1 ± 0.2	13.1 ± 0.1	13.2 ± 0.1	13.1 ± 0.1	13.9 ± 0.3
Day 23	15.2 ± 0.2	14.7 ± 0.2	15.1 ± 0.2	15.0 ± 0.1	14.8 ± 0.1	11.6 ± 0.5**
Week 14	15.0 ± 0.2	14.7 ± 0.1	14.7 ± 0.1	14.9 ± 0.1	13.9 ± 0.1**	11.1 ± 0.3**
Erythrocytes (10^6/µL)						
Day 4	7.03 ± 0.15	6.94 ± 0.09	7.00 ± 0.09	7.08 ± 0.09	7.06 ± 0.05	7.56 ± 0.13**
Day 23	8.05 ± 0.09	7.96 ± 0.08	8.14 ± 0.09	8.13 ± 0.06	8.06 ± 0.06	7.64 ± 0.15
Week 14	8.73 ± 0.11	8.49 ± 0.05	8.70 ± 0.07	8.75 ± 0.05	8.72 ± 0.07	9.45 ± 0.10**
Mean cell volume (fL)						
Day 4	57.9 ± 0.3	57.9 ± 0.1	57.2 ± 0.2	57.6 ± 0.3	56.7 ± 0.4*	55.5 ± 0.3**
Day 23	56.6 ± 0.3	56.3 ± 0.2	55.7 ± 0.2	56.3 ± 0.2	55.1 ± 0.2**	46.7 ± 0.9**
Week 14	51.7 ± 0.2	51.4 ± 0.2	51.0 ± 0.2*	51.0 ± 0.2*	48.9 ± 0.2**	35.6 ± 1.3**
Mean cell hemoglobin (pg)						
Day 4	18.8 ± 0.1	18.9 ± 0.1	18.8 ± 0.1	18.7 ± 0.1	18.5 ± 0.1	18.4 ± 0.1*
Day 23	18.8 ± 0.1	18.4 ± 0.1*	18.5 ± 0.1*	18.5 ± 0.1*	18.4 ± 0.1**	15.1 ± 0.3**
Week 14	17.2 ± 0.1	17.3 ± 0.1	16.9 ± 0.1	17.1 ± 0.1	16.0 ± 0.1**	11.8 ± 0.4**
Mean cell hemoglobin concentration (g/dL)						
Day 4	32.5 ± 0.1	32.6 ± 0.2	32.8 ± 0.1	32.5 ± 0.2	32.7 ± 0.1	33.1 ± 0.2*
Day 23	33.3 ± 0.2	32.7 ± 0.2	33.2 ± 0.2	32.8 ± 0.2	33.3 ± 0.2	32.2 ± 0.2**
Week 14	33.2 ± 0.1	33.7 ± 0.2	33.2 ± 0.1	33.4 ± 0.1	32.6 ± 0.1	33.2 ± 0.2
Platelets (10^3/µL)						
Day 4	983.9 ± 10.7	1,021.1 ± 12.6	973.2 ± 27.6	986.2 ± 22.2	992.8 ± 21.9	1,021.8 ± 20.9
Day 23	886.2 ± 16.6	957.3 ± 13.2**	973.2 ± 25.7**	1,042.7 ± 22.2**	1,133.1 ± 33.0**	1,896.3 ± 58.1**
Week 14	690.7 ± 10.2	755.6 ± 15.4**	777.5 ± 26.3**	912.8 ± 20.2**	1,090.4 ± 30.7**	1,780.3 ± 113.0**
Clinical Chemistry						
n	10	10	10	10	10	10
Alanine aminotransferase (IU/L)						
Day 4	68 ± 1	74 ± 2	71 ± 3	79 ± 2**	84 ± 2**	119 ± 8**
Day 23	55 ± 2	53 ± 2	53 ± 2	59 ± 2	78 ± 4**	92 ± 3**
Week 14	63 ± 6	52 ± 1	51 ± 1	61 ± 3	77 ± 6*	182 ± 20**
Alkaline phosphatase (IU/L)						
Day 4	885 ± 24	962 ± 19	895 ± 23	923 ± 16	887 ± 22	913 ± 34
Day 23	667 ± 13	638 ± 12	622 ± 19	668 ± 17	656 ± 19	673 ± 20
Week 14	261 ± 7	243 ± 3	251 ± 5	269 ± 5	277 ± 8	303 ± 9**
Sorbitol dehydrogenase (IU/L)						
Day 4	18 ± 1	20 ± 0	17 ± 1	18 ± 0	20 ± 1	24 ± 1**
Day 23	24 ± 2	22 ± 1	23 ± 1	25 ± 1	31 ± 3	28 ± 1
Week 14	19 ± 2	14 ± 1	19 ± 1	22 ± 1	23 ± 2	34 ± 2**

TABLE 3
Selected Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male (continued)						
Clinical Chemistry (continued)						
n	10	10	10	10	10	10
Bile salts (μmol/L)						
Day 4	20.5 ± 1.8	23.0 ± 2.7	30.3 ± 2.5**	32.1 ± 2.9**	39.3 ± 3.2**	68.8 ± 12.1**
Day 23	28.5 ± 5.3	20.4 ± 2.1	32.8 ± 2.3	41.4 ± 4.8*	52.2 ± 5.3**	60.2 ± 6.7**
Week 14	15.2 ± 1.4	20.3 ± 1.8*	25.3 ± 2.9**	36.4 ± 4.1**	53.1 ± 6.4**	129.0 ± 11.5**
Iron (μg/dL)						
Week 14	200.1 ± 8.6	179.3 ± 4.5	164.9 ± 5.3**	184.6 ± 5.0	189.1 ± 6.6	161.3 ± 9.2*
Total iron binding capacity (μg/dL)						
Week 14	656.5 ± 12.9	659.7 ± 14.9	654.9 ± 11.8	652.9 ± 6.3	1,034.1 ± 21.7**	1,578.8 ± 43.8**
Female						
Hematology						
n	10	10	10	10	10	10
Hematocrit (%)						
Day 4	42.4 ± 0.7	44.3 ± 0.6	42.8 ± 0.8	43.8 ± 0.8	42.2 ± 0.9	43.1 ± 0.6
Day 23	47.1 ± 0.5	46.5 ± 0.3	47.1 ± 0.6	47.8 ± 0.5	46.8 ± 0.4	42.4 ± 0.5**
Week 14	45.7 ± 0.5	45.4 ± 0.2	45.1 ± 0.5	44.5 ± 0.4	45.6 ± 0.2	41.0 ± 0.4**
Hemoglobin (g/dL)						
Day 4	14.0 ± 0.2	14.6 ± 0.2	14.2 ± 0.2	14.5 ± 0.3	13.8 ± 0.3	14.3 ± 0.2
Day 23	15.6 ± 0.2	15.3 ± 0.1	15.6 ± 0.2	15.7 ± 0.2	15.4 ± 0.1	13.8 ± 0.2**
Week 14	15.2 ± 0.1	15.1 ± 0.1	14.9 ± 0.1	14.7 ± 0.2	15.0 ± 0.1	13.2 ± 0.1**
Erythrocytes (10^6/μL)						
Day 4	7.36 ± 0.12	7.75 ± 0.11	7.50 ± 0.15	7.69 ± 0.15	7.44 ± 0.18	7.74 ± 0.11
Day 23	8.32 ± 0.10	8.29 ± 0.06	8.37 ± 0.11	8.46 ± 0.11	8.41 ± 0.07	8.64 ± 0.09
Week 14	8.39 ± 0.09	8.34 ± 0.05	8.27 ± 0.09	8.16 ± 0.08	8.55 ± 0.04	9.21 ± 0.10**
Mean cell volume (fL)						
Day 4	57.6 ± 0.2	57.1 ± 0.2	57.1 ± 0.3	57.0 ± 0.3	56.7 ± 0.3*	55.6 ± 0.2**
Day 23	56.7 ± 0.1	56.0 ± 0.1**	56.3 ± 0.2*	56.4 ± 0.3*	55.7 ± 0.2**	49.1 ± 0.6**
Week 14	54.5 ± 0.1	54.5 ± 0.1	54.5 ± 0.1	54.5 ± 0.2	53.3 ± 0.1**	44.6 ± 0.7**
Mean cell hemoglobin (pg)						
Day 4	19.0 ± 0.1	18.8 ± 0.1	18.9 ± 0.1	18.8 ± 0.1	18.6 ± 0.1*	18.5 ± 0.0**
Day 23	18.8 ± 0.1	18.5 ± 0.1**	18.6 ± 0.1*	18.6 ± 0.1*	18.4 ± 0.1**	16.0 ± 0.2**
Week 14	18.1 ± 0.1	18.0 ± 0.1	18.0 ± 0.1	18.0 ± 0.1	17.6 ± 0.1**	14.3 ± 0.2**
Mean cell hemoglobin concentration (g/dL)						
Day 4	33.0 ± 0.2	33.0 ± 0.2	33.1 ± 0.2	33.0 ± 0.1	32.9 ± 0.1	33.2 ± 0.1
Day 23	33.2 ± 0.1	33.0 ± 0.2	33.1 ± 0.2	33.0 ± 0.2	33.0 ± 0.1	32.6 ± 0.1**
Week 14	33.3 ± 0.1	33.2 ± 0.1	33.0 ± 0.1	33.0 ± 0.1	33.0 ± 0.1	32.2 ± 0.1**
Platelets (10^3/μL)						
Day 4	881.9 ± 56.8	914.0 ± 46.4	849.8 ± 33.7	961.4 ± 23.7	880.0 ± 14.2	970.2 ± 21.0
Day 23	861.4 ± 15.2	835.5 ± 39.1	859.9 ± 31.1	875.7 ± 35.4	914.7 ± 24.2	1,410.3 ± 45.8**
Week 14	728.2 ± 16.7	763.2 ± 11.9	801.3 ± 16.4**	824.9 ± 10.8**	857.8 ± 20.3**	1,048.1 ± 31.2**

TABLE 3
Selected Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Female (continued)						
Clinical Chemistry						
n	10	10	10	10	10	10
Alanine aminotransferase (IU/L)						
Day 4	60 ± 2	61 ± 3	63 ± 1	63 ± 2	68 ± 2**	109 ± 4**
Day 23	48 ± 2	49 ± 1	49 ± 2	53 ± 1*	61 ± 2**	93 ± 4**
Week 14	49 ± 2	40 ± 2	40 ± 1	45 ± 1	51 ± 1	97 ± 6**
Alkaline phosphatase (IU/L)						
Day 4	790 ± 19	761 ± 16	762 ± 17	743 ± 14	726 ± 10*	765 ± 13
Day 23	497 ± 11	494 ± 13	512 ± 10	517 ± 7	486 ± 13	624 ± 19**
Week 14	222 ± 6	236 ± 10	219 ± 7	229 ± 11	221 ± 6	226 ± 10
Sorbitol dehydrogenase (IU/L)						
Day 4	18 ± 1	17 ± 1	16 ± 1	18 ± 1	18 ± 0	22 ± 2*
Day 23	18 ± 1	18 ± 1	19 ± 1	18 ± 0	18 ± 1	21 ± 2
Week 14	16 ± 1	17 ± 1	16 ± 1	16 ± 1	23 ± 1**	34 ± 2**
Bile salts (µmol/L)						
Day 4	22.7 ± 3.3	18.1 ± 2.3	19.6 ± 1.4	22.4 ± 1.6	23.1 ± 2.3	45.1 ± 2.8**
Day 23	19.2 ± 2.8	23.6 ± 3.0	31.7 ± 3.7	27.0 ± 3.9	33.7 ± 2.7**	73.7 ± 9.9**
Week 14	18.8 ± 1.8	17.9 ± 1.3	25.9 ± 2.2*	24.5 ± 2.5*	32.7 ± 2.2**	53.1 ± 3.8**
Iron (µg/dL)						
Week 14	321.8 ± 9.2	320.1 ± 13.6	308.1 ± 10.9	328.9 ± 29.6	247.1 ± 14.1**	267.3 ± 20.0**
Total iron binding capacity (µg/dL)						
Week 14	597.3 ± 8.7	606.1 ± 7.5	604.8 ± 13.2	591.4 ± 10.1	607.1 ± 9.0	1,151.3 ± 34.1**

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test
** $P \leq 0.01$
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.

Serum markers of iron metabolism were evaluated in the core study rats at week 14. At this timepoint, serum iron concentration was decreased in the 600 mg/kg males and 300 and 600 mg/kg females. Additionally, total iron-binding capacity, an estimator of total transferrin, increased substantially in the 300 and 600 mg/kg males and the 600 mg/kg females. Based on the group mean data, the average saturation of total iron binding capacity decreased from approximately 31% and 54% in vehicle control males and females, respectively, to 10% and 23% in the 600 mg/kg males and females, respectively. An iron profile such as this would be consistent with an iron deficiency. Because there was no evidence of chronic blood loss, the mechanism in this study is unknown.

In general, serum activities of alanine aminotransferase were increased in the 300 and 600 mg/kg males and females throughout the study. By week 14, the increase ameliorated in the 300 mg/kg or less groups, but worsened in the 600 mg/kg groups. Serum sorbitol dehydrogenase activity also demonstrated increases in the 600 mg/kg groups. The increased serum activities of both enzymes would suggest increased hepatocellular leakage and could be consistent with morphological liver changes (e.g., chronic inflammation) observed in this study.

Additionally, serum bile salt concentration demonstrated a dose-related increase in the 75 mg/kg or greater males and 300 mg/kg or greater females and could be consistent with cholestasis. In this study, however, serum alkaline phosphatase activity, another marker of cholestasis, remained fairly stable throughout the study, suggesting that the bile salt increases may not have been related to a cholestatic event. Serum bile salt concentrations can be affected by mechanisms other than cholestasis (e.g., altered enterohepatic circulation, impaired hepatic function, or noncholestatic liver injury that elevates circulating bile salt concentrations) (Hofmann, 1988). In contrast, serum alkaline phosphatase activity increases minimally in response to hepatocellular damage (Hoffmann *et al.*, 1989).

A dose-related decrease in serum albumin, and hence total protein concentrations, occurred in dosed males and females. The decreases were of minimal severity and ameliorated with time. The mechanism for this transient effect is unknown, but may be associated with the morphological liver changes as a result of an alteration (e.g., reduction) in hepatic protein synthesis (e.g., albumin) or possibly related to a decreased nutritional status (e.g., decreased food intake) as evidenced by the decreased body weights.

Absolute and relative liver weights of all dosed groups were increased compared to those of the vehicle control groups, and the increases were statistically significant for 300 and 600 mg/kg males and for 75 mg/kg or greater females (Tables 4 and D1). Relative kidney weights were significantly increased in all dosed groups of male rats and in 75 mg/kg or greater females. The absolute and relative testis weights of 300 and 600 mg/kg males were significantly decreased. Relative lung weights of all dosed groups of males were significantly greater than those of the vehicle controls, but differences in absolute lung weights were not dose related.

TABLE 4
Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
n	10	10	10	10	10	10
Male						
Necropsy body wt	338 ± 5	348 ± 5	342 ± 4	328 ± 5	285 ± 5**	242 ± 4**
R. Kidney						
Absolute	0.993 ± 0.027	1.122 ± 0.016**	1.094 ± 0.031*	1.073 ± 0.023	1.017 ± 0.030	0.987 ± 0.019
Relative	2.943 ± 0.074	3.230 ± 0.047**	3.198 ± 0.070**	3.270 ± 0.061**	3.565 ± 0.054**	4.073 ± 0.065**
Liver						
Absolute	13.475 ± 0.267	14.494 ± 0.247	13.876 ± 0.337	14.321 ± 0.346	18.920 ± 0.821**	27.226 ± 0.900**
Relative	39.952 ± 0.768	41.716 ± 0.733	40.568 ± 0.579	43.609 ± 0.702	66.204 ± 1.875**	112.264 ± 3.042**
Lung						
Absolute	1.494 ± 0.045	1.720 ± 0.042	1.708 ± 0.044	1.672 ± 0.078	1.493 ± 0.040	1.286 ± 0.052**
Relative	4.428 ± 0.130	4.954 ± 0.133*	5.001 ± 0.127*	5.110 ± 0.267*	5.265 ± 0.196**	5.308 ± 0.197**
R. Testis						
Absolute	1.338 ± 0.027	1.413 ± 0.022	1.366 ± 0.021	1.458 ± 0.060	0.949 ± 0.039**	0.554 ± 0.015**
Relative	3.970 ± 0.090	4.067 ± 0.071	3.997 ± 0.036	4.443 ± 0.177	3.337 ± 0.139**	2.289 ± 0.058**
Female						
Necropsy body wt	196 ± 3	199 ± 4	197 ± 3	192 ± 2	179 ± 3**	169 ± 3**
R. Kidney						
Absolute	0.666 ± 0.014	0.698 ± 0.018	0.719 ± 0.013	0.696 ± 0.012	0.688 ± 0.013	0.709 ± 0.016
Relative	3.407 ± 0.057	3.506 ± 0.054	3.655 ± 0.051**	3.633 ± 0.048**	3.842 ± 0.046**	4.207 ± 0.079**
Liver						
Absolute	6.561 ± 0.206	7.083 ± 0.169	7.488 ± 0.168**	7.913 ± 0.112**	8.607 ± 0.181**	15.420 ± 0.284**
Relative	33.540 ± 0.811	35.622 ± 0.809	38.043 ± 0.520**	41.335 ± 0.574**	48.025 ± 0.539**	91.689 ± 2.036**

* Significantly different (P≤0.05) from the vehicle control group by Williams' test
** Significantly different (P≤0.01) from the vehicle control group by Williams' or Dunnett's test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

Gross lesions were observed in the liver of both sexes and the kidney of male rats; the lesions were observed primarily in the 300 and 600 mg/kg groups. Gross lesions of the liver included mottled discoloration, enlargement (males), increased granular appearance, and tan discoloration of the right anterior and posterior lobes (males). The kidneys of 600 mg/kg males had focal dark discolorations.

Two 600 mg/kg male rats had multiple cholangiocarcinomas in the liver; a third had a hepatocellular adenoma (Tables 5 and A1). The hepatocellular adenoma was a large nodular mass with distinct borders and compression of the adjacent liver parenchyma (Plate 2). It consisted of mildly to moderately pleomorphic hepatocytes that were normal in size or slightly larger than normal and arranged in abnormal lobular patterns. The hepatic cords within an adenoma usually intersected the surrounding normal hepatic cords at an oblique or right angle. A few portal triads or bile ducts were found at the periphery of the lesion; these likely represented entrapped structures. The cholangiocarcinomas were irregular, relatively large, multiple lesions composed of numerous irregular and atypical bile ducts in a matrix of fibrous connective tissue (Plate 3). The atypical bile ducts were discontinuous, often having crescent shapes, and lined by large, very basophilic, cuboidal to columnar epithelial cells with degenerative changes and frequent mitotic figures. Stratification of these epithelial cells was present in some areas. The atypical bile ducts frequently contained mucinous material and cellular debris. The stroma of these tumors consisted of abundant actively proliferating fibrous connective tissue. The frequent presence of neoplastic atypical biliary epithelium within the adjacent normal hepatic parenchyma was evidence of localized invasion. There was no evidence of metastases to regional lymph nodes or other sites by the two cholangiocarcinomas.

All 600 mg/kg males had cholangiofibrosis. These were multiple lesions that varied greatly in size with a few large lesions effacing most of one or more liver lobes. All were locally expansive, proliferative lesions having irregular shapes and consisting of atypical bile ducts surrounded by prominent, dense, collagenous stroma infiltrated by a variety of chronic inflammatory cells. The ducts were formed by large, cuboidal to squamoid, often hyperchromatic basophilic cells. The ductular lumens often contained mucinous material, necrotic cell debris, and a mixture of inflammatory cells. The majority of cholangiofibrosis lesions were small, located in portal areas, and tended to have a more mature fibrous connective tissue component and less atypia of the epithelial cells than the larger lesions. The smaller size and the clear separation of these lesions from adjacent liver parenchyma distinguished cholangiofibrosis from cholangiocarcinoma (Plate 4). With larger lesions, the distinction was less evident and there appeared to be a morphological continuum from cholangiofibrosis to cholangiocarcinoma.

All males given 75 mg/kg or greater and all females given 150 mg/kg or greater had hepatocellular hypertrophy, and the severity increased with increasing dose (Tables 5, A1, and A2). Hepatocellular hypertrophy was characterized by large hepatocytes having increased amounts of cytoplasm. This hypertrophy was accompanied by increased numbers of hepatocytes, multinucleated hepatocytes, and mitotic figures. These hepatocytic changes were distributed

TABLE 5
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
Number Examined Microscopically	10	10	10	10	10	10
Hepatocyte, Hypertrophy[a]	0	0	10** (1.0)[b]	10** (2.0)	10** (3.0)	10** (4.0)
Bile Duct, Hyperplasia	0	10** (1.0)	10** (1.0)	10** (1.0)	10** (2.0)	10** (2.0)
Oval Cell, Hyperplasia	0	10** (1.0)	10** (1.1)	10** (2.0)	10** (3.0)	10** (3.0)
Periportal, Inflammation, Chronic	0	10** (1.0)	10** (1.0)	10** (2.0)	10** (2.0)	10** (2.0)
Cholangiofibrosis	0	0	0	0	0	10** (2.6)
Basophilic Focus	0	0	0	6**	9**	6**
Mixed Cell Focus	0	0	1	9**	10**	8**
Eosinophilic Focus	0	0	0	1	10**	10**
Clear Cell Focus	0	0	0	3	1	1
Periportal, Infiltration Cellular, Histiocyte	0	10** (1.0)	10** (1.1)	10** (2.0)	10** (2.0)	10** (2.0)
Hepatocellular Adenoma	0	0	0	0	0	1
Cholangiocarcinoma, Multiple	0[c]	0	0	0	0	2
Female						
Number Examined Microscopically	10	10	10	10	10	10
Hepatocyte, Hypertrophy	0	0	0	10** (1.0)	10** (2.0)	10** (4.0)
Bile Duct, Hyperplasia	0	10** (1.0)	10** (1.0)	10** (1.0)	10** (2.0)	10** (2.0)
Oval Cell, Hyperplasia	0	10** (1.0)	10** (1.0)	10** (1.0)	10** (2.0)	10** (3.0)
Periportal, Inflammation, Chronic	0	5* (1.0)	10** (1.0)	10** (1.0)	10** (2.0)	10** (2.0)
Basophilic Focus	0	0	3	6**	10**	10**
Mixed Cell Focus	0	0	1	6**	10**	10**
Eosinophilic Focus	0	0	0	0	0	10**
Clear Cell Focus	0	0	0	0	0	1
Periportal, Infiltration Cellular, Histiocyte	0	0	0	10** (1.0)	10** (2.0)	10** (2.0)

* Significantly different (P≤0.05) from the vehicle control group by the Fisher exact test
** P≤0.01
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Significant trend (P≤0.01) by the Cochran-Armitage test

throughout the lobules. Sometimes entire lobules consisted of hypertrophic hepatocytes. Often the livers were irregularly multinodular and resembled nodular regeneration. This appearance resulted from an accentuated lobular architecture due to partial or complete perilobular proliferations of fibrous connective tissue that often extended across adjacent lobules (Plate 4).

Incidences of bile duct hyperplasia, oval cell hyperplasia, and chronic periportal inflammation of the liver were significantly increased in all dosed groups, and the severities of these lesions were generally increased in the 300 and 600 mg/kg groups. Bile duct hyperplasia consisted of multifocal proliferations of small bile ducts within portal areas. These bile ducts were similar in appearance to those seen in portal triads of normal liver lobules. The only distinctive

feature was increased numbers of ducts. They consisted of uniform basophilic, flattened to cuboidal epithelium surrounded by scant to moderate amounts of collagenous stroma. Oval cell hyperplasia consisted of proliferations of small spindle to oval cells extending along the hepatic sinusoids from the periportal zones. Oval cells were uniform in size with scant basophilic cytoplasm and indistinct margins. Chronic periportal inflammation consisted of mixed infiltrates with small numbers of mononuclear inflammatory cells within portal areas.

Incidences of basophilic focus were increased in 75 mg/kg females while both basophilic and mixed cell foci were significantly increased in rats administered 150 mg/kg or greater. Incidences of eosinophilic focus were significantly increased in 300 and 600 mg/kg males and 600 mg/kg females. Clear cell foci occurred in a few exposed rats. These eosinophilic, mixed, basophilic, and clear cell foci were similar in appearance but consisted of hepatocytes with altered tinctorial properties. In an eosinophilic focus, at least 80% of the cells had eosinophilic cytoplasm. The mixed cell focus was composed of a mixture of cells with different staining properties, generally having less than 20% eosinophilic cells and 80% or more clear cells. Combinations with basophilic cells were uncommon. A basophilic focus consisted of hepatocytes with a prominent basophilic cytoplasm, occasionally having linear intracytoplasmic aggregates. When the cells had a clear cytoplasm, it was termed a clear cell focus. Other than tinctorial differences, foci consisted of hepatocytes that were generally somewhat larger than those in the adjacent parenchyma but otherwise were similar in appearance and arranged in relatively normal lobular patterns. The hepatic cords at the periphery of these foci generally merged imperceptibly with the surrounding normal liver, resulting in an indistinct border and little or no compression of the adjacent liver parenchyma.

Incidences of cellular infiltration of the periportal region of the liver by histiocytes (macrophages containing refractile gray, tan, or dusky pigment) were significantly increased in all 150 mg/kg or greater rats, and the severity was increased in the higher dosed groups.

Incidences of bone marrow hyperplasia were increased in 150 mg/kg males and significantly increased in 75, 300, and 600 mg/kg males (Tables 6 and A1). Hyperplasia was generally of mild severity at 600 mg/kg and minimal at lower doses, consisting of increased numbers of erythroid series hematopoietic cells within the marrow cavity.

Incidences of renal tubule papillary mineralization were increased in 150 mg/kg males and were significantly increased in 300 and 600 mg/kg males; incidences of papillary mineralization were increased in 75, 100 and 300 mg/kg females and significantly increased in 600 mg/kg females (Tables 6, A1, and A2). Incidences of cortical renal tubule pigmentation were significantly increased in 150 mg/kg or greater males, and the severity increased at 600 mg/kg. The incidence of renal tubule regeneration was significantly increased in 600 mg/kg females. Renal papillary mineralization was generally of minimal severity and occurred as linear granular deposits or concretions

TABLE 6
Incidences of Selected Nonneoplastic Lesions in Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
Bone Marrow[a]	10	10	10	10	10	10
Hyperplasia[b]	0	0	4* (1.0)[c]	3 (1.0)	10** (1.0)	10** (2.0)
Kidney	10	10	10	10	10	10
Papilla, Renal Tubule, Mineralization	4 (1.0)	2 (1.0)	2 (1.0)	8 (1.0)	10** (1.0)	10** (1.9)
Cortex, Renal Tubule, Pigmentation	0	0	0	10** (1.0)	10** (1.0)	10** (2.0)
Nose	10	10	10	10	10	10
Olfactory Epithelium, Degeneration	0	0	0	0	9** (1.3)	10** (2.1)
Pituitary Gland	10	10	10	10	10	10
Pars Distalis, Chromophobe Cell, Hypertrophy	0	0	0	0	10** (1.8)	10** (1.6)
Salivary Gland	10	10	10	10	10	10
Submandibular Gland, Cytoplasmic Alteration	0	0	10** (1.0)	10** (1.0)	10** (2.0)	10** (2.0)
Stomach, Glandular	10	10	10	10	10	10
Epithelium, Glands, Atrophy	0	0	2 (1.0)	10** (1.0)	10** (1.7)	10** (2.2)
Testes	10	10	10	10	10	10
Bilateral, Germinal Epithelium, Degeneration	0	0	0	0	10** (3.9)	10** (4.0)
Epididymis	10	10	10	10	10	10
Bilateral, Hypospermia	0	0	0	0	10** (3.4)	10** (4.0)
Female						
Kidney	10	10	10	10	10	10
Papilla, Renal Tubule, Mineralization	3 (1.0)	4 (1.0)	6 (1.0)	7 (1.0)	9** (1.0)	7 (1.0)
Renal Tubule, Regeneration	1 (1.0)	2 (1.0)	1 (1.0)	1 (1.0)	3 (1.0)	6* (1.0)
Nose	10	10	10	10	10	10
Olfactory Epithelium, Degeneration	0	0	1 (1.0)	2 (1.0)	10** (1.6)	10** (2.1)
Salivary Gland	10	10	10	10	10	10
Submandibular Gland, Cytoplasmic Alteration	0	0	10** (1.0)	10** (1.0)	10** (2.0)	10** (2.0)
Stomach, Glandular	10	10	10	10	10	10
Epithelium, Glands, Atrophy	0	0	0	8** (1.0)	10** (1.3)	10** (2.0)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

primarily within the cytoplasm or in the lumens of the collecting tubules and occasionally the cortical and medullary tubules. Renal cortical tubule epithelial pigmentation occurred as round or oval refractile tan or brown bodies in the cytoplasm or free in the lumens of cortical renal tubules. Kidneys with cortical tubule pigmentation from five 600 mg/kg males were examined with Perl's stain for hemosiderin, which contains ferric iron. They were negative for ferric iron, indicating that the pigment was not hemosiderin. Renal tubule regeneration was consistent with the early stages of chronic progressive nephropathy and consisted of multifocal, scattered small foci of cortical tubules lined by increased numbers of low cuboidal epithelial cells with lightly basophilic cytoplasm.

Incidences of degeneration of the olfactory epithelium in the nose were significantly increased in 300 and 600 mg/kg males and females (Tables 6, A1, and A2). This lesion occurred in Levels II and III of the dorsal meatus and consisted of decreased height and disorganization of the olfactory epithelium. The degeneration was accompanied by disorderly proliferation of the basal epithelial cells, loss of Bowman's glands, and atrophy of the olfactory nerve bundles adjacent to affected segments of olfactory epithelium.

Incidences of hypertrophied chromophobe cells in the pars distalis of the pituitary gland were significantly increased in 300 and 600 mg/kg males (Tables 6 and A1). The pars distalis of affected pituitary glands had increased numbers of vacuolated, lightly basophilic, enlarged cells that frequently contained a large perinuclear clear zone or vacuole. The vacuolated cells were morphologically similar to those described as "castration cells" that are promoted by reduced hormonal secretions from the testes.

Cytoplasmic alteration (decreased cytoplasmic granulation) in the serous portion of the submandibular salivary gland occurred in all 75 mg/kg or greater rats (Tables 6, A1, and A2). This lesion consisted of mild decreases in the size of cells of the serous acini and marked decreases in the size and prominence of secretory convoluted (granular) ducts of the submandibular salivary glands due to loss of cytoplasmic zymogen granules.

Incidences of atrophy of the gastric glands in the stomach were significantly increased at 150 mg/kg or greater, and severities increased with increasing dose (Tables 6, A1, and A2). The diagnosis of atrophy included a spectrum of changes in the fundic region of the glandular stomach, the most conspicuous of which was decreased height of the mucosal glands due to loss of parietal cells and chief cells. Other associated changes were scattered individual parietal and chief cell necrosis, minimal to mild infiltrates of polymorphonuclear inflammatory cells within the lamina propria, and occasional dilation of the base of the glands (Plates 5, 6, and 7).

Incidences of bilateral degeneration of the germinal epithelium in the testes and bilateral hypospermia of the epididymis were significantly increased in 300 and 600 mg/kg males (Tables 6 and A1). Degeneration affected seminiferous tubules, which were devoid of germinal epithelial cells and lined only by Sertoli cells (Plates 8 and 9). Epididymal hypospermia was invariably associated with testicular degeneration and consisted of complete absence or markedly reduced numbers of mature spermatozoa within the tubules.

Stomach-related findings for the special study rats on day 31 are reported in Table 7. Serum gastrin concentration and stomach pH were significantly increased in 600 mg/kg rats. Microscopic findings for the stomachs after 30 days of exposure were similar to those reported after 3 months of exposure. At 30 days, significantly increased incidences of atrophy of the gastric glands in the fundic region of the glandular stomach were seen at 300 and 600 mg/kg (Table 7). In 300 mg/kg rats, the atrophy included glandular dilatation with flattened epithelium at the bases of

TABLE 7
Stomach Results for Special Study Rats Treated with Estragole by Gavage for 30 Days[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
n	10	10	10	10	10	10
Serum Gastrin (pg/mL)	213.9 ± 31.1	144.3 ± 19.9	137.9 ± 30.9	213.1 ± 39.3	292.7 ± 37.6	3,058.2 ± 277.4**
Stomach pH	5.280 ± 0.286	5.920 ± 0.221	5.720 ± 0.084	5.490 ± 0.224	5.480 ± 0.259	6.710 ± 0.302**
Stomach, Glandular Epithelium, Glands, Atrophy[b]	0	0	0	0	10** (1.0)[c]	10** (2.0)
Female						
n	10	10	10	9	10	10
Serum Gastrin (pg/mL)	225.6 ± 27.4	211.4 ± 25.7	206.1 ± 22.5	215.6 ± 86.9	393.3 ± 79.7	1,863.0 ± 60.5**
Stomach pH	5.150 ± 0.174	5.280 ± 0.148	5.540 ± 0.126	5.067 ± 0.164	5.160 ± 0.186	6.150 ± 0.289*
Stomach, Glandular Epithelium, Glands, Atrophy	0	0	0	0	10** (1.0)	10** (2.0)

* Significantly different (P≤0.05) from the vehicle control group by Dunn's (stomach pH) test
** Significantly different (P≤0.01) from the vehicle control group by Shirley's (serum gastrin), Dunn's (stomach pH), or the Fisher exact (lesions) test
[a] Data (serum gastrin and stomach pH) are presented as mean ± standard error. Samples were collected on day 31.
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

glands. In 600 mg/kg rats, the glandular changes included atrophy with dilatation and epithelial degeneration, necrosis, hyperplasia, and increased infiltrations by a variety of inflammatory cells, predominantly polymorphonuclear cells. These changes resulted in combinations of atrophy, degeneration, and disorganization of the fundic glandular epithelium (Plate 6). At 300 mg/kg, the basal part of the fundic glands was primarily affected. At 600 mg/kg, disorganization involved entire glands.

The results of cytochrome P450 measurements in special study rats are listed in Table 8. Cytochrome P450 1A1-(CYP1A1)-associated 7-ethoxyresorufin-*O*-deethylase (EROD), CYP1A2-associated acetanilide-4-hydroxylase (A4H), and CYP2B-associated 7-pentoxyresorufin-*O*-deethylase (PROD) activities were determined in microsomal proteins isolated from liver tissue according to established procedures. CYP1A2-associated A4H activity was significantly increased in 150 mg/kg or greater female rats; however, the difference was small.

TABLE 8
Cytochrome P450 Data for Special Study Rats Treated with Estragole by Gavage for 30 Days[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
n	10	10	10	10	10	10
Acetanilide-4-hydroxylase (A4H) (nmol/minute per mg microsomal protein)	0.514 ± 0.022	0.559 ± 0.031	0.579 ± 0.023	0.609 ± 0.038	0.638 ± 0.029	0.431 ± 0.026
7-Ethoxyresorufin-*O*-deethylase (EROD) (pmol/minute per mg microsomal protein)	31.921 ± 1.064	38.800 ± 3.468	37.661 ± 1.368	35.469 ± 0.572	39.213 ± 2.132	27.250 ± 2.479
7-Pentoxyresorufin-*O*-deethylase (PROD) (pmol/minute per mg microsomal protein)	4.856 ± 0.161	6.798 ± 0.357**	7.239 ± 0.183**	18.169 ± 0.607**	26.769 ± 1.685**	19.746 ± 1.479**
Female						
n	9	10	10	9	10	10
Acetanilide-4-hydroxylase (A4H) (nmol/minute per mg microsomal protein)	0.553 ± 0.022[b]	0.612 ± 0.027	0.600 ± 0.022[c]	0.717 ± 0.049**	0.759 ± 0.040**	0.689 ± 0.048**
7-Ethoxyresorufin-*O*-deethylase (EROD) (pmol/minute per mg microsomal protein)	44.961 ± 1.182	47.689 ± 1.858	54.717 ± 1.761**	59.140 ± 3.968**	65.182 ± 3.404**	51.640 ± 2.393**
7-Pentoxyresorufin-*O*-deethylase (PROD) (pmol/minute per mg microsomal protein)	4.274 ± 0.198	4.379 ± 0.235	5.146 ± 0.180*	12.436 ± 0.681**	26.070 ± 2.185**	22.694 ± 1.770**

* Significantly different (P≤0.05) from the vehicle control group by Shirley's test
** P≤0.01
[a] Data are presented as mean ± standard error. Samples were collected on day 31.
[b] n=8
[c] n=9

CYP1A1-associated EROD activity was significantly increased in 75 mg/kg or greater females; however, the magnitude of induction was small. In contrast, PROD activity was significantly increased in all dosed groups of male and female rats, except for the 37.5 mg/kg females. The increase in activity generally increased with dose and showed that estragole induced hepatic CYP2B in males and females.

3-Month Study in Mice

One 600 mg/kg male mouse died during week 9, and all 600 mg/kg female mice died during week 1 due to liver necrosis; mean body weights and mean body weight gains of 300 and 600 mg/kg males and surviving females in the 75 mg/kg and greater groups were significantly less than those of the vehicle control groups (Table 9; Figure 4). No exposure-related clinical findings were observed in surviving mice; mice that died early were lethargic and exhibited reduced locomotor and exploratory behavior.

Table 9
Survival and Body Weights of Mice in the 3-Month Gavage Study of Estragole

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight[c] Relative to Controls (%)
		Initial	Final[c]	Change	
Male					
0	10/10	22.2 ± 0.3	36.1 ± 1.0	13.8 ± 0.8	
37.5	10/10	22.5 ± 0.3	36.3 ± 1.0	13.9 ± 0.8	101
75	10/10	22.2 ± 0.3	37.2 ± 1.3	15.0 ± 1.1	103
150	10/10	22.5 ± 0.2	34.5 ± 0.5	12.0 ± 0.4	96
300	10/10	22.5 ± 0.4	30.6 ± 0.8**	8.1 ± 0.5**	85
600	9/10[d]	22.4 ± 0.4	28.4 ± 0.5**	6.0 ± 0.5**	79
Female					
0	10/10	18.3 ± 0.2	29.8 ± 0.6	11.5 ± 0.5	
37.5	10/10	18.1 ± 0.3	28.7 ± 0.8	10.7 ± 0.7	97
75	10/10	18.0 ± 0.3	26.6 ± 0.7**	8.6 ± 0.6**	89
150	10/10	18.2 ± 0.2	25.7 ± 0.3**	7.5 ± 0.3**	86
300	10/10	18.2 ± 0.2	24.3 ± 0.3**	6.1 ± 0.4**	82
600	0/10[e]	18.5 ± 0.2	—	—	—

** Significantly different (P≤0.01) from the vehicle control group by Williams' test
[a] Number of animals surviving at 3 months/number initially in group
[b] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to day 85 of the study.
[c] Day 85
[d] Week of death: 9
[e] Week of deaths: 1

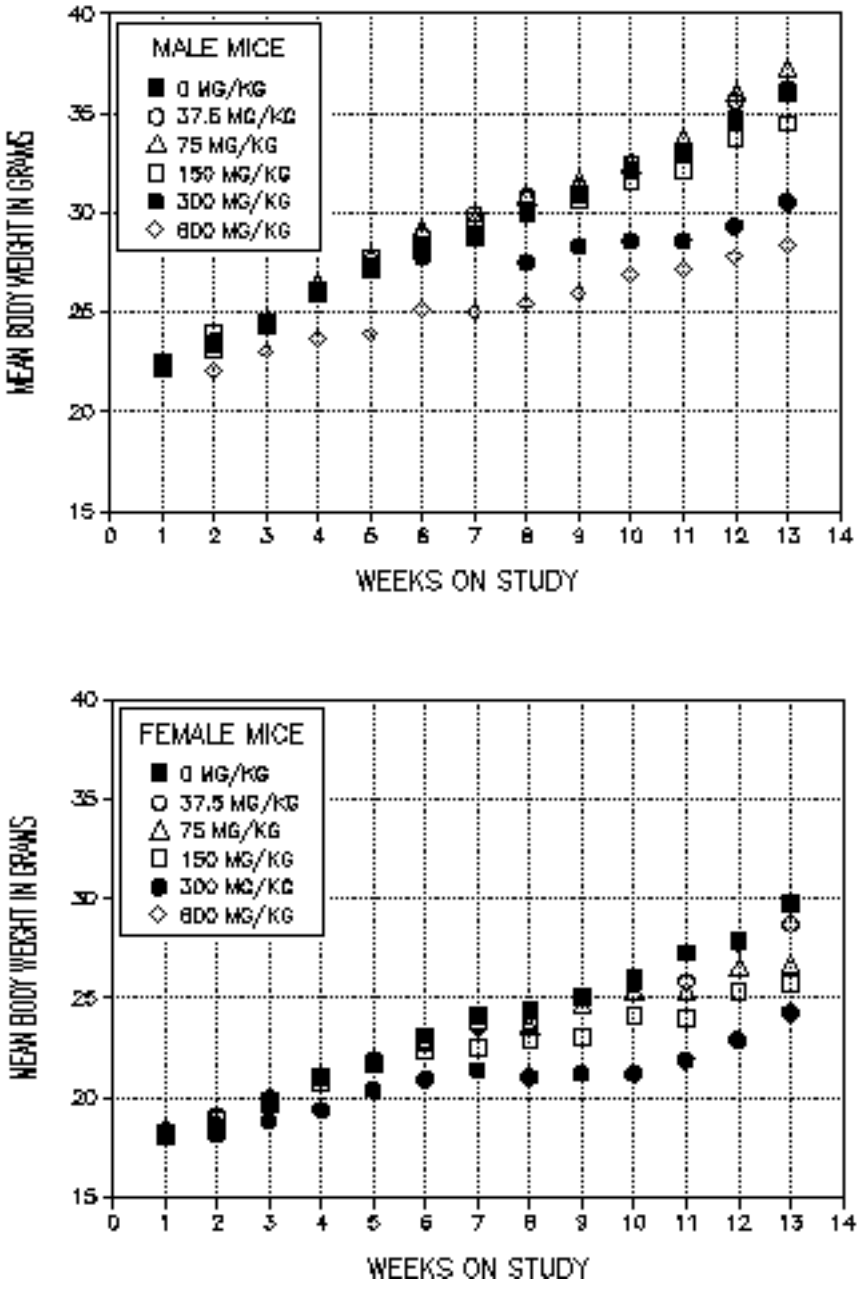


FIGURE 4
Growth Curves for Male and Female Mice Administered Estragole by Gavage for 3 Months

Estragole had little effect on hematology parameters in mice that were exposed at the same levels used in the rat study (Table C2). In fact, the only apparent erythron change in the mice was a slight (≤3%) increase in mean cell volume. The leukocyte profile in mice was similar to that observed in the rat study. Estimated white cell counts from blood smears did not confirm these differences.

Absolute liver weights were increased in all dosed groups of males, and the increases were significant in the 75 and 150 mg/kg groups; absolute liver weight was also significantly increased in 300 mg/kg females (Tables 10 and D2). Relative liver weights were significantly increased in 75 mg/kg or greater groups.

TABLE 10
Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
n	10	10	10	10	10	9
Necropsy body wt	36.6 ± 1.1	37.1 ± 1.0	37.8 ± 1.3	34.8 ± 0.4	31.2 ± 0.8**	28.8 ± 0.4**
Liver						
Absolute	1.546 ± 0.051	1.639 ± 0.044	1.793 ± 0.077*	1.784 ± 0.049*	1.660 ± 0.060	1.676 ± 0.048
Relative	42.276 ± 0.667	44.194 ± 0.762	47.398 ± 0.731**	51.181 ± 0.964**	53.108 ± 0.768**	58.160 ± 1.167**
Female						
n	10	10	10	10	10	0
Necropsy body wt	29.4 ± 0.7	27.5 ± 0.8*	26.2 ± 0.8**	24.8 ± 0.2**	24.3 ± 0.3**	
Liver						
Absolute	1.286 ± 0.026	1.178 ± 0.041	1.213 ± 0.033	1.273 ± 0.010	1.558 ± 0.029**	
Relative	43.949 ± 1.099	42.829 ± 0.597	46.503 ± 0.763*	51.263 ± 0.450**	64.188 ± 1.084**	

* Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

While incidences of hepatocellular hypertrophy and hepatocellular degeneration were increased in 75 mg/kg females, they were increased significantly in 300 and 600 mg/kg males and 150 and 300 mg/kg females (Tables 11, A3, and A4). Severities of these lesions increased with increasing dose. Hypertrophy was characterized by patchy, centrilobular hepatocyte cytomegaly and usually karyomegaly. Affected hepatocytes had abundant coarse eosinophilic cytoplasm. Eosinophilic pseudoinclusions were common in enlarged cells. Hepatocellular degeneration affected randomly scattered individual hepatocytes. Affected hepatocytes had dense hyperchromatic eosinophilic cytoplasm and condensed or fragmented nuclei (apoptotic cells) and occasionally the contracted cytoplasm was globular and surrounded by a clear space (apoptotic bodies).

TABLE 11
Incidences of Selected Nonneoplastic Lesions in Mice in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
Liver[a]	10	10	10	10	10	10
Hepatocyte, Degeneration[b]	0	0	0	0	5* (1.0)[c]	10** (1.6)
Hepatocyte, Hypertrophy	0	0	0	0	8** (1.1)	10** (2.7)
Oval Cell, Hyperplasia	0	0	0	0	10** (1.0)	9** (2.3)
Nose	10	10	10	10	10	10
Olfactory Epithelium, Degeneration	0	0	1 (2.0)	2 (1.0)	10** (1.2)	10** (1.6)
Female						
Liver	10	10	10	10	10	10
Hepatocyte, Degeneration	0	1 (1.0)	3 (1.0)	10** (1.5)	10** (2.0)	0
Hepatocyte, Hypertrophy	0	0	3 (1.0)	10** (2.0)	10** (2.9)	0
Oval Cell, Hyperplasia	0	1 (1.0)	10** (1.0)	10** (2.0)	10** (2.7)	5* (1.4)
Necrosis	0	0	0	0	0	10** (3.4)
Fatty Change, Diffuse	0	0	0	0	0	5* (1.6)
Stomach, Glandular	10	10	10	10	10	10
Epithelium, Glands, Degeneration	0	0	0	0	1 (1.0)	5* (1.6)
Stomach, Forestomach	10	10	10	9	10	10
Hyperplasia, Squamous	0	0	0	0	0	6** (1.5)
Mineralization	0	0	0	0	0	6** (1.8)
Ulcer	0	0	0	0	0	6** (2.0)
Nose	10	10	10	10	10	10
Olfactory Epithelium, Degeneration	0	0	0	2 (1.0)	10** (1.1)	10** (3.4)

* Significantly different (P≤0.05) from the vehicle control group by the Fisher exact test
** P≤0.01
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

Incidences of oval cell hyperplasia in the liver were significantly increased in 300 and 600 mg/kg males and in 75, 150, 300, and 600 mg/kg females. Oval cell hyperplasia consisted of increased numbers of small basophilic cells with oval nuclei and scant cytoplasm proliferating along and lining the hepatic sinusoids. Minimal or mild oval cell hyperplasia was generally limited to periportal areas. When the hyperplasia was moderate to marked, proliferating oval cells diffusely lined the hepatic sinusoids and in some cases formed sheets of cells. In the severest instances, proliferating oval cells disrupted the hepatic architecture and largely obscured other hepatic lesions.

Liver necrosis occurred in all 600 mg/kg female mice that died during week 1. This lesion was related to estragole exposure and was considered to be the cause of death. Five 600 mg/kg females also had diffuse fatty change, which indicated further hepatocellular toxicity caused by estragole. Necrosis varied from moderate to marked among affected animals and consisted of widespread single cell coagulative hepatocellular necrosis associated with minimal mononuclear inflammatory cell infiltrates. Diffuse fatty change was characterized by numerous clear microvacuoles within the cytoplasm of hepatocytes that were consistent with the presence of fat.

Along with the liver changes, 600 mg/kg females exhibited significant increases in the incidences of degeneration of the gastric glands of the glandular stomach and squamous hyperplasia, mineralization, and ulcers in the forestomach (Tables 11 and A4). Degeneration of the gastric glands consisted of degeneration, necrosis and loss of the parietal cells of the gastric mucosal glands in the fundic portion of the stomach with shortening of the glands and atrophy of the mucosa. Affected cells had hyperchromatic eosinophilic and granular cytoplasm with fragmented nuclei (karyorrhexis). Some affected cells had contracted cytoplasm surrounded by a clear space (karyolysis). Ulcers were characterized by loss of the mucosal epithelium, with necrosis, mineralization, and chronic active inflammation of the superficial lamina propria. Ulcers were accompanied by minimal to mild thickening of the forestomach epithelium (squamous epithelial hyperplasia).

Incidences of degeneration of the olfactory epithelium in the nose were significantly increased in 300 and 600 mg/kg mice (Tables 11, A3, and A4). Degeneration of the olfactory epithelium was characterized by loss of cilia, loss of the normal apical eosinophilic cytoplasm, accumulation of eosinophilic intracytoplasmic material, loss of cellular polarity (nuclei disorganized with respect to normal stratification within the epithelium), and apoptosis or single cell necrosis. The nerve fiber layer was thinned proportionately to the extent of alteration and cell loss in overlying olfactory epithelium. There was also segmental replacement of the olfactory epithelium by columnar ciliated epithelial cells (respiratory epithelial metaplasia) that often contained large cytoplasmic hyaline droplets. The general appearance of the affected olfactory epithelium was segmental mucosal atrophy, denudation, and loss and disorganization of the remaining epithelial cells.

The deaths of 600 mg/kg female mice during the first week of exposure were associated with atrophy of lymphoid follicles and cellular depletion of the red pulp in the spleen, atrophy of the mandibular and mesenteric lymph nodes, and marked lymphoid necrosis in the thymus (Table A4). These findings were considered to be nonspecific terminal events associated with glucocorticoid release and were considered secondary to liver necrosis. Atrophy of the splenic lymphoid follicles and of the mesenteric and mandibular lymph nodes was characterized by a decreased cellularity from the loss of a variety of lymphoid cells. Cellular depletion of the splenic red pulp resulted from generalized loss of cells, including lymphocytes, macrophages, granulocytes, plasma cells, and erythrocytes. After 3 to 5 days of exposure, the thymus of the female mice had marked lymphoid necrosis with blending of the boundaries between the cortex and medulla. In both the cortex and medulla, necrosis resulted in massive destruction of thymic lymphocytes. Many of the remaining thymocytes had small, shrunken, dense nuclei. Much of the lymphoid tissue was replaced by intensely basophilic granular fragments having various sizes or fused irregular clumps of basophilic nuclear and cytoplasmic debris. The epithelial tissues of the medulla and the macrophages were less severely affected.

GENETIC TOXICOLOGY

Estragole (1 to 220 µg/plate) was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 when tested with or without rat or hamster liver metabolic activation enzymes (Table B1; Zeiger *et al.*, 1987). No significant increases in the frequencies of micronucleated normochromatic erythrocytes were observed in peripheral blood samples from male and female mice in the 3-month study, and no significant changes in percent polychromatic erythrocytes were seen, indicating no estragole-induced toxicity to bone marrow (Table B2).

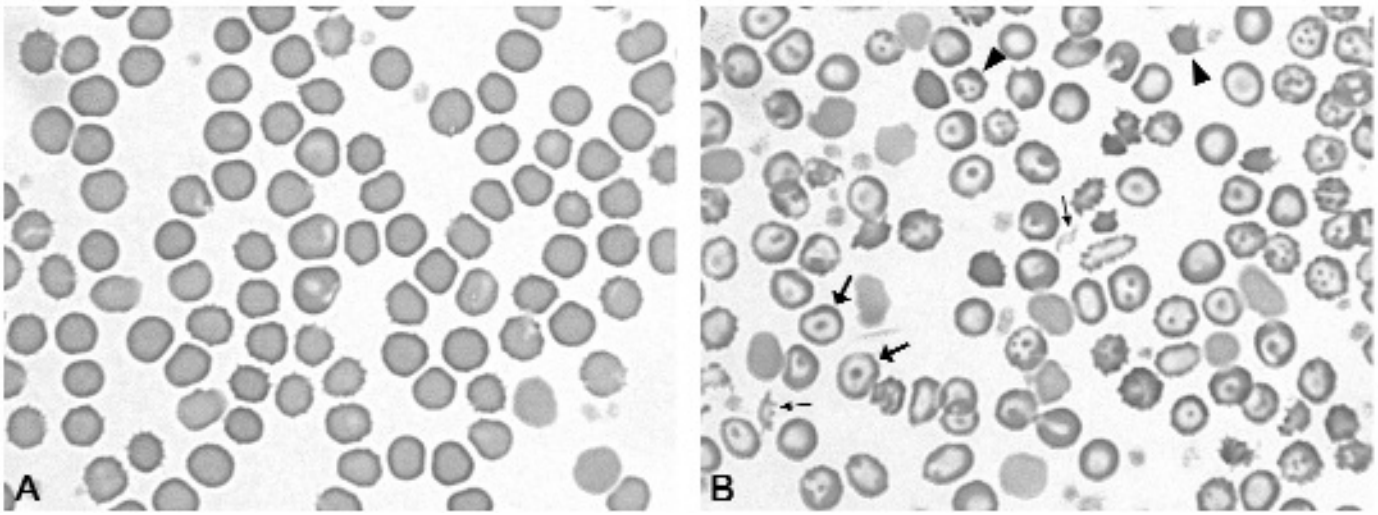


Plate 1
Blood film photomicrographs of vehicle control (A) and 600 mg/kg (B) male F344/N rats at week 14 from the 3-month gavage study of estragole. In 600 mg/kg males and females, there was an increase in the overall central pallor of the erythrocytes and increased incidences of target cells (thick arrows), erythrocyte fragments (thin arrows), and microcytes (arrow heads). Wright's Stain

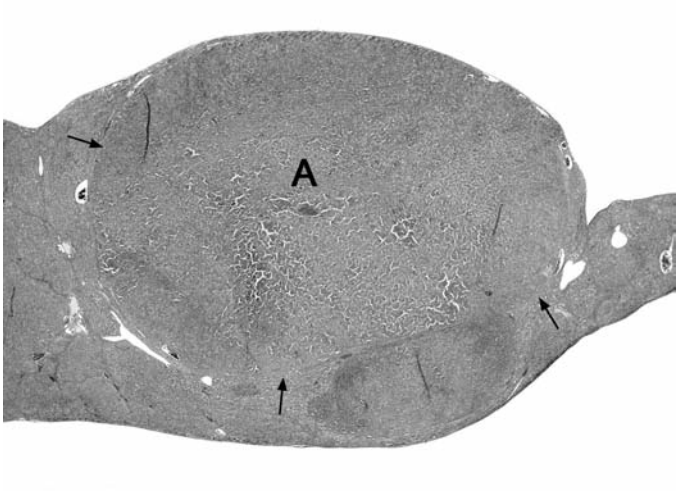


Plate 2
Large hepatocellular adenoma (A) in the liver of a male F344/N rat administered 600 mg/kg estragole by gavage for 3 months. The adenoma has replaced most of the affected lobe. Note that this neoplastic mass has distinct boundaries (arrows) that compress the adjacent liver parenchyma. H&E

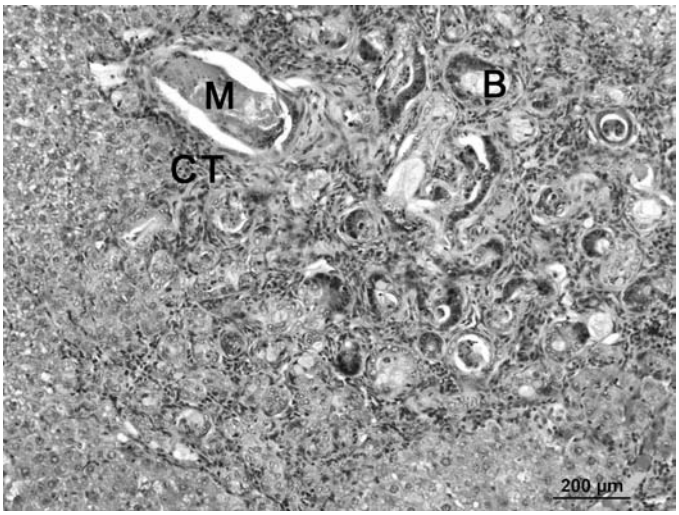


Plate 3
Cholangiocarcinoma in the liver of a male F344/N rat administered 600 mg /kg estragole by gavage for 3 months. Note the characteristic neoplastic atypical bile ducts (B) and fibrous connective tissue (CT) proliferation. These bile ducts are formed by multiple layers of pleomorphic, basophilic epithelial cells and have little resemblance to normal bile ducts. Mucus accumulation (M) is variable and inflammatory cells are often present. H&E

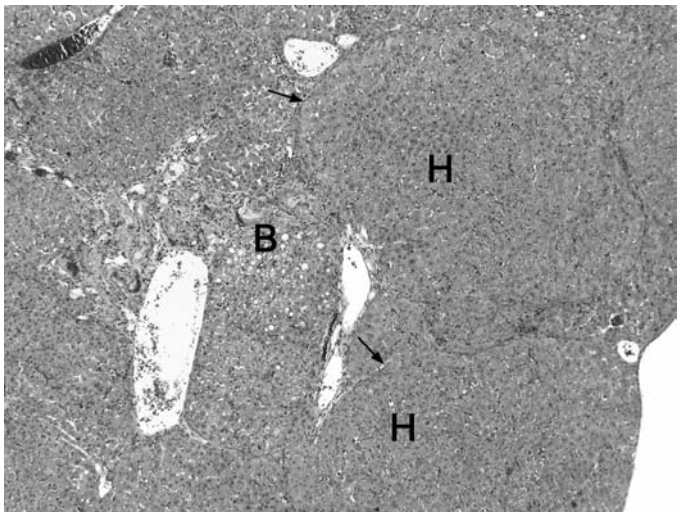


Plate 4
Mild cholangiofibrosis (B) and moderate hepatocytic hypertrophy (H) in the liver of a male F344/N rat administered 600 mg/kg estragole by gavage for 3 months. Note the multiple nodular areas of hepatocytic hypertrophy (arrows) throughout the liver. H&E

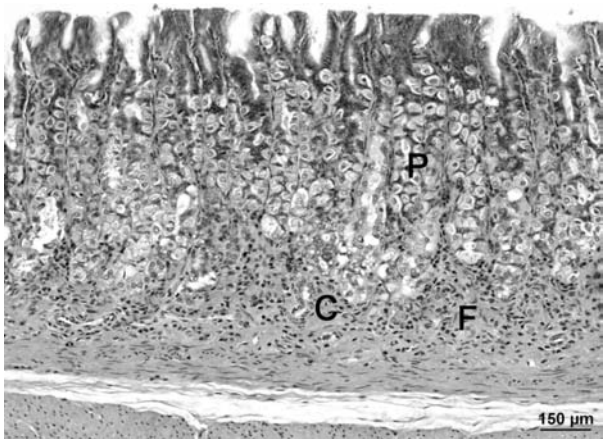


Plate 5
Atrophy in the glandular stomach of a male F344/N rat administered 600 mg/kg estragole by gavage for 3 months. Note the increased fibrous connective tissue (F) in the lamina propria below the gastric glands. The glands have lost the orderly progression in cell types and are decreased in length. Many of the parietal (P) and chief cells (C) are missing. Degeneration of parietal cells has resulted in cytoplasmic swelling and loss of the characteristic eosinophilic cytoplasmic granules. Compare with normal glandular stomach in the male F344/N vehicle control rat shown in Plate 7. H&E

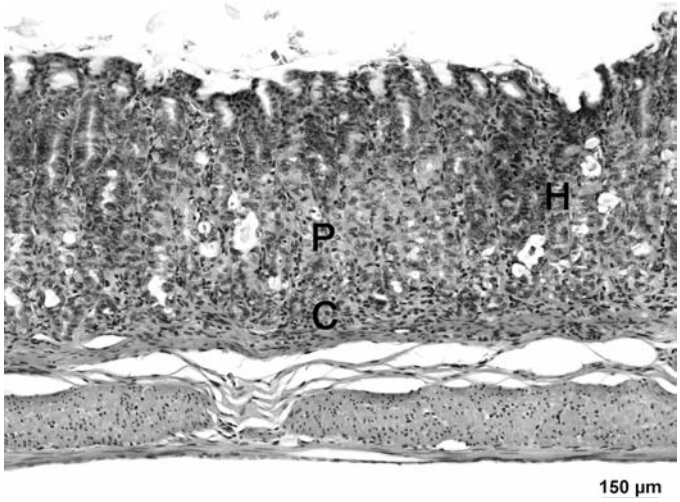


Plate 6
Atrophy in the glandular stomach of a male F344/N special study rat administered 600 mg/kg estragole by gavage for 30 days. The gastric glands are disorganized, atrophic, dysplastic, and dilated with degeneration of parietal (P) and chief cells (C). Epithelium lining the glands is often replaced by hyperplastic (H) and dysplastic cells. H&E

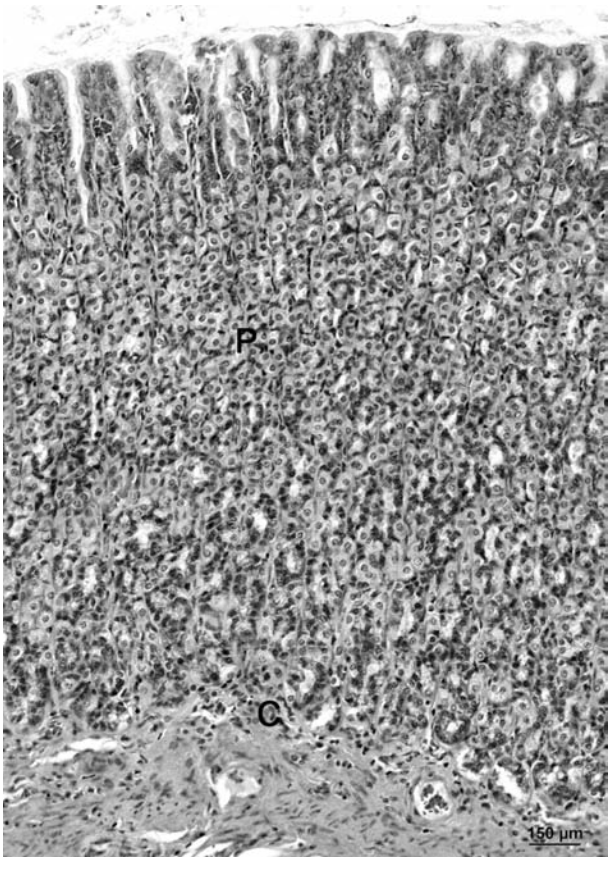


Plate 7
Normal glandular stomach in a male F344/N vehicle control rat at 3 months in the gavage study of estragole. Note the orderly progression of the epithelial cells in the gastric glands with midzonal parietal (P) and basal chief cells (C) and limited amounts of fibrous connective tissue in the lamina propria. H&E

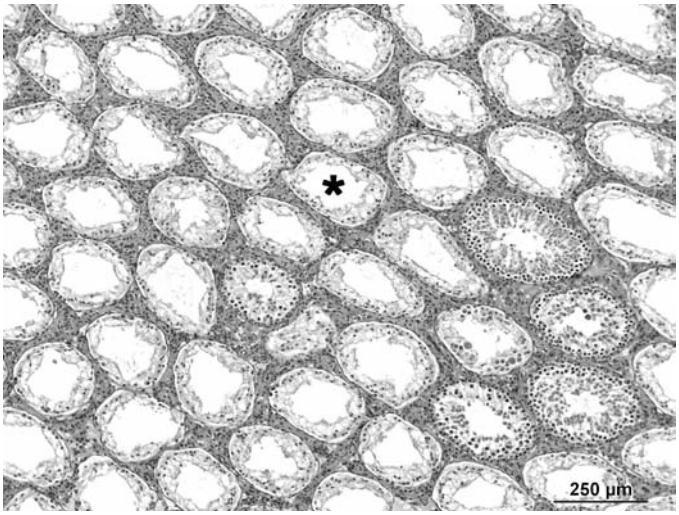


Plate 8
Marked degeneration of the germinal epithelium in the testis of a male F344/N rat administered 600 mg/kg estragole by gavage for 3 months. Note the lack of spermatogenesis (asterisk) in these seminiferous tubules. The predominant cells in the most affected tubules appear to be Sertoli cells. Compare with normal testicular seminiferous tubules in the male F344/N vehicle control rat shown in Plate 9. H&E

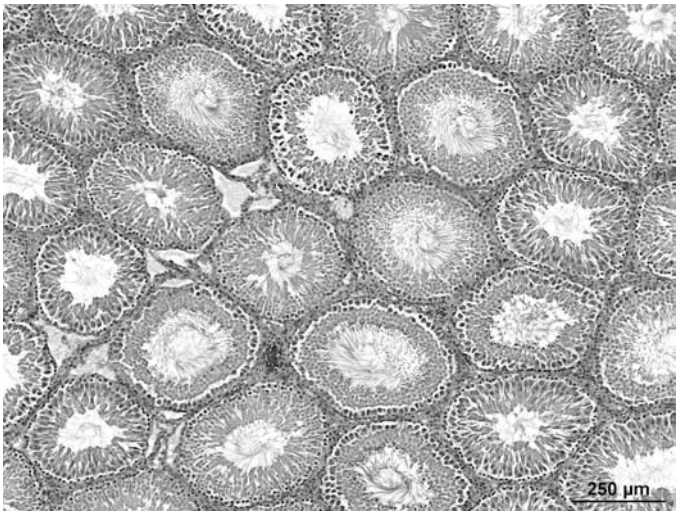


Plate 9
Normal spermatogenesis in the testicular seminiferous tubules of a male F344/N vehicle control rat at 3 months in the gavage study of estragole. H&E

DISCUSSION

Estragole is a high production volume chemical that is added to foods as a flavoring substance and is used as a fragrance in cleaning agents and cosmetic products. As a result, there is a high potential for human exposure. Numerous studies have established that estragole is an hepatocarcinogen in multiple mouse strains following oral, intraperitoneal, or subcutaneous exposure (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987). However, there is no good evidence of carcinogenicity in rats. Estragole is structurally related to the allylbenzene analogues, methyleugenol and safrole, which are carcinogenic in both rats and mice. Estragole is converted to its corresponding 1-hydroxy and 1-sulfate ester conjugates, the putative proximate and ultimate carcinogenic metabolites, respectively, which are formed by metabolism of allylbenzene analogues. The purpose of the current studies was to better characterize the subchronic toxicity and potential carcinogenicity of estragole in F344/N rats and B6C3F1 mice. In order to make direct comparisons to National Toxicology Program (NTP) studies of methyleugenol (NTP, 2000) and isoeugenol (NTP, 2010), gavage was chosen as the route of administration and comparable doses and endpoints were evaluated. The highest dose of 600 mg/kg in the estragole study was selected based on the significant toxicity to rats or mortality of mice administered 1,000 mg/kg in the 3-month studies of methyleugenol (NTP, 2000). The same gavage doses were used for the 3-month studies of estragole and isoeugenol.

All rats survived to the end of dosing in the estragole study; however, all 600 mg/kg female mice died during week 1 due to liver necrosis. Similar toxicity was not observed in rats and mice administered 600 mg/kg isoeugenol for 3 months (NTP, 2010). In the present study, mean body weights were significantly decreased in rats and male mice administered 300 or 600 mg/kg and female mice administered 75 mg/kg or greater. Primary target organs identified for rats administered estragole included liver, glandular stomach, nose, kidney, and salivary gland for males and females. Estragole-related toxicity was also observed in the bone marrow, pituitary gland, testes, and epididymides of male rats. In mice, the liver and nose of males and females and the stomach of females were identified as target organs of estragole toxicity. A comparison of the present study results with those of the 3-month (Abdo *et al.*, 2001) and 2-year studies of methyleugenol (Johnson *et al.*, 2000; NTP, 2000) illustrates the similarities and differences in toxic effects of these phenylpropenoid compounds.

Alterations in the erythron related to estragole administration occurred in male and female rats; males exhibited a stronger response. The changes in the erythron corresponded to a microcytic, normochromic, nonresponsive anemia and were consistent with ineffective erythropoiesis. Additionally, dose-related increases in platelet counts occurred

in most dosed groups of rats. Similar changes in the erythron and platelets were observed in the 3-month methyleugenol study (NTP, 2000).

In the 3-month estragole study, the liver was a target organ of estragole administration in both rats and mice. Liver weights were generally increased in both species. In rats, neoplasms and nonneoplastic lesions in the liver were attributed to estragole treatment. Two 600 mg/kg male rats had multiple cholangiocarcinomas; an additional male rat had an hepatocellular adenoma. A search of concurrent NTP subchronic study results showed that there were no liver neoplasms of any type in 662 control males and 677 control females in 3-month studies (NTP, 2007). The two cholangiocarcinomas in the present 3-month study exhibited an unusual form of this neoplasm that has only been reported after chemical exposure during 2-year studies. They differed from spontaneous cholangiomas and cholangiocarcinomas that are solitary lesions having irregular branching glandular structures lined by a single layer of well-differentiated epithelium in benign lesions or single to multiple layers of malignant epithelial cells with frequent mitotic figures, high nuclear to cytoplasmic ratios, pleomorphism and anisokaryosis. The fibrous connective tissue component of the spontaneous cholangiolar tumors is minimal and consists of dense collagen and relatively few benign fibrocytes. The chemically induced cholangiocarcinomas and cholangiofibrosis are multifocal lesions having atypical bile duct epithelium surrounded by abundant actively growing connective tissue and can displace or invade adjacent liver parenchyma.

All 600 mg/kg male rats had cholangiofibrosis, which is considered to be a morphological continuum with cholangiocarcinoma. Biological information relative to the pathogenesis or progression of these lesions is limited. As a result, the most appropriate classification scheme for these lesions is somewhat uncertain and controversial. It was clear that small, benign appearing lesions warranted the nonneoplastic diagnosis of cholangiofibrosis. The cholangiocarcinomas appeared aggressive with apparent invasion into adjacent liver tissues and warranted the diagnosis of a malignant lesion. Lesions in one animal that did not readily fit the criteria for cholangiocarcinoma posed a diagnostic challenge and were given the diagnosis of marked cholangiofibrosis. Other chemicals that have resulted in chemical-related cholangiocarcinomas or cholangiofibrosis similar to those observed in the present study include the 2-year study of furan (Maronpot *et al.*, 1991; NTP, 1993) and several 2-year studies conducted as part of dioxin toxic equivalency factor (TEF) evaluations, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), and mixtures containing these chemicals (NTP, 2006a,b,c,d,e,f). Notably, in the 3-month study of furan, cholangiofibrosis was observed in nearly all males and females in the four highest dosed groups (NTP, 1993). In the 2-year study of furan, cholangiocarcinomas occurred in all groups of rats and was present in many male and female rats at the 9- and 15-month interim evaluations (NTP, 1993). Hepatocellular neoplasms of the liver were also significantly increased in rats and mice.

In the 3-month study of estragole, bile duct hyperplasia, oval cell hyperplasia, and periportal chronic inflammation were observed in the liver of all dosed male and female rats, providing additional evidence that the bile duct epithelium is a major target of estragole. Additionally, periportal histiocytic infiltrates were increased significantly in all dosed groups of males and in 150 mg/kg or greater females. Increased incidences of oval cell hyperplasia also occurred in 300 and 600 mg/kg male mice and 75 mg/kg or greater female mice. The presence of an hepatocellular adenoma, cellular infiltration of the periportal region by histiocytes, as well as increased incidences of basophilic, eosinophilic, and mixed cell foci in rats, coupled with moderate to marked necrosis that caused deaths in the 600 mg/kg female mice, indicate that the hepatocytes are also a target of estragole. Hepatocellular injury caused by estragole was evidenced by increases in serum alanine aminotransferase and sorbitol dehydrogenase activities and dose-related increases in serum bile acid concentrations in dosed rats. Liver lesions were observed at lower doses in the 3-month study of estragole than in the 3-month study of methyleugenol (NTP, 2000).

Until now, there was no evidence that estragole was carcinogenic in the rat. The literature provides strong evidence that estragole is a liver carcinogen in mice (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987). Estragole induced liver tumors in multiple strains and both sexes of mice following at least three different routes of exposure. Two closely related compounds, safrole and methyleugenol, are hepatocarcinogenic in rats. Notably, in the 3-month study of methyleugenol, one hepatocellular adenoma was observed in a male rat administered 1,000 mg/kg (Abdo *et al*, 2001; NTP, 2000). Also, after 12-months of dosing with 300 mg/kg of methyleugenol, hepatocellular adenoma was diagnosed in four male rats (including two with multiple hepatocellular adenomas), another had hepatocholangiocarcinoma, and hepatocellular carcinoma was observed in one female rat (Johnson *et al.*, 2000; NTP, 2000). The mode of action for estragole carcinogenicity and other allylbenzene analogues has been well characterized and occurs through a genotoxic mechanism. Estragole is bioactivated in the liver to 1-hydroxyestragole which is conjugated with sulfate to form an electrophilic sulfate ester that binds to DNA, RNA, and proteins. Bioactivation of estragole also results in the formation of potentially reactive epoxide metabolites. The 1-hydroxy metabolite of estragole was shown to be a stronger hepatocarcinogen than the parent compound in mice (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987). 1-Hydroxyestragole was generally positive in mutagenicity assays, induced unscheduled DNA synthesis, and produced several DNA adduct species in the livers of mice. The magnitude of DNA adduct formation in preweanling mice exposed to estragole, safrole, and methyleugenol correlated well with liver tumor incidences (Phillips *et al.*, 1984). In brachymorphic mice or mice that are preexposed to a sulfotransferase inhibitor, adduct formation and carcinogenicity is inhibited (Boberg *et al*, 1983; Randerath *et al.*, 1984; Wiseman *et al.*, 1987), providing additional evidence that the 1-hydroxy and 1-sulfate ester metabolites are responsible for the genotoxicity and carcinogenicity of estragole.

Because conversion of allylbenzene analogues to their mutagenic/procarcinogenic 1-hydroxy metabolites is catalyzed by cytochrome P450 enzyme systems (Ioannides *et al.*, 1981; Gardner *et al.*, 1997; Jeurissen *et al.*, 2004, 2006, 2007), CYP1A1, 1A2, and 2B activities were assayed in livers of rats exposed to estragole for 30 days. Studies using various specific P450 isozyme inhibitors indicated that in control rat liver microsomes, 1-hydroxylation of methyleugenol is catalyzed predominantly by CYP 2E1 and probably CYP 2C6 (Gardner *et al.*, 1997). When P450 activities were measured in liver microsomes isolated from rats that were exposed *in vivo* to methyleugenol, CYP2B (PROD) and 1A2 (A4H) were found to be induced, along with lesser induced CYPs (Gardner *et al.*, 1997). Like methyleugenol, estragole induced an approximately 5-fold increase in CYP2B-associated PROD activity in both male and female rats. However, only small increases in CYP 1A2 activity were observed in female rats. Jeurissen *et al.* (2007) identified and measured the activity of P450 enzymes in human liver that convert estragole, safrole, and methyleugenol to their 1-hydroxy metabolites. While all isozymes tested, except CYP 2C8, showed some activity, CYP 1A2 and 2A6 were most efficient in the 1-hydroxylation of estragole. They also demonstrated that methyleugenol and safrole, although structurally similar, are converted to their 1-hydroxy metabolites by different P450 enzymes; methyleugenol is bioactivated by CYP 1A2 and safrole is bioactivated by CYP 2A6. Competition for P450 enzymes may occur when combinations of these allylbenzene analogues are present in the liver.

In this 3-month study of estragole, toxicity was observed in the stomach of rats and female mice. In the NTP studies of methyleugenol, gastric pH and serum gastrin levels were significantly increased in female F344/N rats after 30 or 90 days of exposure; male B6C3F1 mice were less sensitive to these effects (NTP, 2000). Following 2-years of methyleugenol administration, incidences of neuroendocrine tumors of the glandular stomach in dosed male and female rats were increased; two male mice also had gastric neuroendocrine tumors. Similar to methyleugenol, dosing with 600 mg/kg estragole for 30 days resulted in significant increases in gastric pH and serum gastrin levels in male and female F344/N rats. Increased incidences of glandular stomach atrophy were observed at 150 mg/kg or greater estragole in male and female rats; severities increased with increasing dose. In rats, glandular stomach toxicity was characterized by atrophy, single-cell necrosis of parietal and chief cells, dilation of the base of glands, and increased infiltration of polymorphonuclear cells. In mice, degeneration of the gastric glands of the glandular stomach occurred only in 600 mg/kg females; gastric pH and serum gastrin levels were not evaluated. In contrast, 30 days of isoeugenol dosing decreased intragastric pH in females and had no effect on serum gastrin levels (NTP, 2010). There was no indication of glandular neuroendocrine hyperplasia or neoplasia in the stomachs of rats or mice exposed to isoeugenol for 2 years. Taken together, these results suggest that estragole may induce tumors in the glandular stomach of rats after longer exposure.

Estragole at 300 or 600 mg/kg for 3 months induced significant increases in mild to moderate olfactory epithelial degeneration in rats and mice. Degeneration or atrophy of the olfactory epithelium, respectively, in rats and mice were observed in the 3-month methyleugenol and isoeugenol studies (NTP, 2000, 2010). However, nasal lesions were absent in the 2-year study of methyleugenol at doses ranging from 37.5 to 150 mg/kg. Nonneoplastic nasal lesions were observed in the 2-year study of isoeugenol, no nasal neoplasms were observed.

Estragole-related toxicity was observed in the pituitary gland, testes, and epididymides of male rats exposed to 300 or 600 mg/kg. Testis weights of 300 and 600 mg/kg males were significantly decreased. All males in these groups had marked bilateral degeneration of the germinal epithelium in the testes and bilateral hypospermia in the epididymides. These changes were most likely due to a direct effect of estragole on the testes resulting in a typical response of the hypothalamic-pituitary-testicular axis including the presence of "castration cells" in the pituitary gland secondary to reduced hormonal secretions from the testes. An indirect effect, for example on Leydig cell hormonal or androgen levels, would have produced a different pattern of cellular changes within the seminiferous tubules to that reported. Cytoplasmic alteration in the salivary gland of males and females also indicated an endocrine effect of estragole. The severity of the testicular and epididymal effects indicates that functional deficits are likely in these males. This study provides evidence that estragole has the potential to be a reproductive toxicant in male rats. Testicular degeneration was also observed in 1,000 mg/kg male rats in the 3-month methyleugenol study, but no significant differences in sperm counts, motility, or morphology were observed at lower doses (NTP, 2000).

Under the conditions of these 3-month studies, estragole showed carcinogenic activity based on the occurrence of two cholangiocarcinomas and one hepatocellular adenoma in the liver of three of 10 male F344/N rats in the high dose group. Because rats and mice were exposed for only 3 months, these studies do not assess the full carcinogenic potential of estragole.

Nonneoplastic effects were observed in the liver, glandular stomach, nose, kidney, and salivary gland of male and female rats and in the testes, epididymides, and pituitary gland of male rats. Nonneoplastic effects were also observed in the liver and nose of male and female mice and in the stomach of female mice.

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APPENDIX A
SUMMARY OF NEOPLASMS AND NONNEOPLASTIC LESIONS IN RATS AND MICE

TABLE A1
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male Rats in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Esophagus	(10)					(10)
Inflammation, chronic						1 (10%)
Intestine large, rectum	(10)					(10)
Parasite metazoan	1 (10%)					
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Angiectasis, focal					2 (20%)	
Basophilic focus				6 (60%)	9 (90%)	6 (60%)
Cholangiofibrosis						10 (100%)
Cholangiocarcinoma, multiple						2 (20%)
Clear cell focus				3 (30%)	1 (10%)	1 (10%)
Eosinophilic focus				1 (10%)	10 (100%)	10 (100%)
Hepatocellular adenoma						1 (10%)
Hepatodiaphragmatic nodule	1 (10%)	3 (30%)		1 (10%)	3 (30%)	
Inflammation, chronic	1 (10%)	2 (20%)	1 (10%)	1 (10%)	3 (30%)	
Mixed cell focus			1 (10%)	9 (90%)	10 (100%)	8 (80%)
Bile duct, hyperplasia		10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Centrilobular, vacuolization cytoplasmic	1 (10%)					
Hepatocyte, hypertrophy			10 (100%)	10 (100%)	10 (100%)	10 (100%)
Oval cell, hyperplasia		10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Periportal, infiltration cellular, histiocyte		10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Periportal, inflammation, chronic		10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Pancreas	(10)					(10)
Acinus, atrophy						1 (10%)
Salivary glands	(10)	(10)	(10)	(10)	(10)	(10)
Submandibular gland, cytoplasmic alteration			10 (100%)	10 (100%)	10 (100%)	10 (100%)
Stomach, glandular	(10)	(10)	(10)	(10)	(10)	(10)
Mineralization				1 (10%)		
Epithelium, glands, atrophy			2 (20%)	10 (100%)	10 (100%)	10 (100%)
Cardiovascular System						
Heart	(10)					(10)
Infiltration cellular, mononuclear cell	2 (20%)					3 (30%)
Myocardium, infiltration cellular, mononuclear cell	6 (60%)					3 (30%)
Endocrine System						
Pituitary gland	(10)	(10)	(10)	(10)	(10)	(10)
Pars distalis, chromophobe cell, hypertrophy					10 (100%)	10 (100%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A1
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
General Body System						
None						
Genital System						
Epididymis	(10)	(10)	(10)	(10)	(10)	(10)
Hypospermia	1 (10%)					
Bilateral, hypospermia					10 (100%)	10 (100%)
Testes	(10)	(10)	(10)	(10)	(10)	(10)
Bilateral, germinal epithelium, degeneration					10 (100%)	10 (100%)
Germinal epithelium, degeneration	1 (10%)			2 (20%)		
Germinal epithelium, mineralization				1 (10%)		
Hematopoietic System						
Bone marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hyperplasia			4 (40%)	3 (30%)	10 (100%)	10 (100%)
Lymph node					(3)	(3)
Pancreatic, hyperplasia, lymphoid					2 (67%)	3 (100%)
Pancreatic, infiltration cellular, histiocyte					3 (100%)	3 (100%)
Lymph node, mesenteric	(10)	(10)	(10)	(10)	(7)	(10)
Hyperplasia, lymphoid		1 (10%)			2 (29%)	2 (20%)
Infiltration cellular, histiocyte				1 (10%)	1 (14%)	1 (10%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)	(10)	(10)	(10)	(10)	(10)
Hamartoma		1 (10%)				
Inflammation, chronic	5 (50%)	3 (30%)	6 (60%)	7 (70%)	6 (60%)	8 (80%)
Inflammation, granulomatous		1 (10%)			4 (40%)	
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Inflammation, chronic				2 (20%)		
Olfactory epithelium, degeneration					9 (90%)	10 (100%)
Special Senses System						
None						

TABLE A1
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Urinary System						
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Infiltration cellular, lymphocyte	1 (10%)		1 (10%)	2 (20%)	7 (70%)	
Cortex, renal tubule, pigmentation				10 (100%)	10 (100%)	10 (100%)
Medulla, renal tubule, mineralization			1 (10%)			
Papilla, renal tubule, mineralization	4 (40%)	2 (20%)	2 (20%)	8 (80%)	10 (100%)	10 (100%)
Renal tubule, cyst			1 (10%)			
Renal tubule, regeneration	7 (70%)	7 (70%)	10 (100%)	10 (100%)	10 (100%)	8 (80%)

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Basophilic focus			3 (30%)	6 (60%)	10 (100%)	10 (100%)
Cholangiofibrosis						1 (10%)
Clear cell focus						1 (10%)
Eosinophilic focus						10 (100%)
Hepatodiaphragmatic nodule	2 (20%)	2 (20%)	2 (20%)	1 (10%)	2 (20%)	
Inflammation, chronic	4 (40%)	4 (40%)	5 (50%)	6 (60%)	3 (30%)	
Mixed cell focus			1 (10%)	6 (60%)	10 (100%)	10 (100%)
Bile duct, hyperplasia		10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Hepatocyte, hypertrophy				10 (100%)	10 (100%)	10 (100%)
Oval cell, hyperplasia		10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Periportal, infiltration cellular, histiocyte				10 (100%)	10 (100%)	10 (100%)
Periportal, inflammation, chronic		5 (50%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Mesentery				(1)		
Accessory spleen				1 (100%)		
Fat, necrosis				1 (100%)		
Salivary glands	(10)	(10)	(10)	(10)	(10)	(10)
Submandibular gland, cytoplasmic alteration			10 (100%)	10 (100%)	10 (100%)	10 (100%)
Stomach, glandular	(10)	(10)	(10)	(10)	(10)	(10)
Mineralization				1 (10%)	2 (20%)	
Necrosis, focal		1 (10%)				
Epithelium, glands, atrophy				8 (80%)	10 (100%)	10 (100%)
Cardiovascular System						
Heart	(10)					(10)
Infiltration cellular, mononuclear cell						1 (10%)
Myocardium, infiltration cellular, mononuclear cell	2 (20%)					2 (20%)
Endocrine System						
None						
General Body System						
None						
Genital System						
Uterus	(10)					(10)
Endometrium, hyperplasia, cystic	3 (30%)					

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Hematopoietic System						
Bone marrow	(10)					(10)
Hyperplasia	1 (10%)					
Lymph node						(4)
Pancreatic, hyperplasia, lymphoid						4 (100%)
Pancreatic, infiltration cellular, histiocyte						4 (100%)
Lymph node, mesenteric	(10)	(10)	(10)	(10)	(10)	(10)
Hyperplasia, lymphoid						3 (30%)
Infiltration cellular, histiocyte				1 (10%)		1 (10%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)	(10	(10)	(10)	(10)	(10)
Inflammation, chronic	5 (50%)	6 (60%)	3 (30%)	4 (40%)	8 (80%)	10 (100%)
Bronchiole, bronchus, hyperplasia, lymphoid			1 (10%)			
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory epithelium, degeneration			1 (10%)	2 (20%)	10 (100%)	10 (100%)
Special Senses System						
Harderian gland	(10)					(10)
Hyperplasia, focal						1 (10%)
Urinary System						
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Infiltration cellular, lymphocyte					1 (10%)	3 (30%)
Cortex, renal tubule, mineralization	2 (20%)	1 (10%)	4 (40%)	4 (40%)	1 (10%)	1 (10%)
Medulla, renal tubule, mineralization					1 (10%)	
Papilla, renal tubule, mineralization	3 (30%)	4 (40%)	6 (60%)	7 (70%)	9 (90%)	7 (70%)
Renal tubule, regeneration	1 (10%)	2 (20%)	1 (10%)	1 (10%)	3 (30%)	6 (60%)
Renal tubule, epithelium, regeneration			1 (10%)			

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early death						
Moribund						1
Survivors						
Terminal sacrifice	10	10	10	10	10	9
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Gallbladder	(10)					(10)
Infiltration cellular, mixed cell	1 (10%)					
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Eosinophilic focus						1 (10%)
Hematopoietic cell proliferation	1 (10%)					
Inflammation, chronic active	2 (20%)	2 (20%)	4 (40%)	4 (40%)	7 (70%)	7 (70%)
Hepatocyte, degeneration					5 (50%)	10 (100%)
Hepatocyte, hypertrophy					8 (80%)	10 (100%)
Oval cell, hyperplasia					10 (100%)	9 (90%)
Salivary glands	(10)				(10)	(10)
Cytoplasmic alteration						1 (10%)
Stomach, forestomach	(10)	(10)	(10)	(10)	(10)	(10)
Hyperplasia, squamous						1 (10%)
Inflammation						1 (10%)
Mineralization	1 (10%)					
Stomach, glandular	(10)	(10)	(10)	(10)	(10)	(10)
Dilatation		1 (10%)				
Infiltration cellular, polymorphonuclear	3 (30%)					
Mineralization					1 (10%)	
Epithelium, glands, atrophy						1 (10%)
Epithelium, glands, degeneration						1 (10%)
Glands, ectasia						1 (10%)
Cardiovascular System						
None						
Endocrine System						
Adrenal cortex	(10)					(10)
Subcapsular, hyperplasia	5 (50%)					6 (60%)
Parathyroid gland	(10)					(8)
Cyst						1 (13%)
General Body System						
None						

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Genital System						
Preputial gland	(10)					(10)
Atrophy						1 (10%)
Ectasia	1 (10%)					
Seminal vesicle	(10)					(10)
Atrophy						1 (10%)
Hematopoietic System						
Bone marrow	(10)					(10)
Atrophy						1 (10%)
Lymph node, mandibular	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy						1 (10%)
Lymph node, mesenteric	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy						1 (10%)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Hematopoietic cell proliferation	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	8 (80%)
Lymphoid follicle, atrophy						1 (10%)
Thymus	(10)					(10)
Atrophy						1 (10%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
Brain	(10)					(10)
Venule, infiltration cellular, mixed cell						1 (10%)
Respiratory System						
Lung	(10)					(10)
Congestion, diffuse						1 (10%)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Inflammation						1 (10%)
Olfactory epithelium, degeneration			1 (10%)	2 (20%)	10 (100%)	10 (100%)
Trachea	(10)					(10)
Inflammation	1 (10%)					
Special Senses System						
Harderian gland	(10)					(10)
Infiltration cellular, mononuclear cell						1 (10%)

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Urinary System						
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Nephropathy	3 (30%)	5 (50%)	2 (20%)	4 (40%)	4 (40%)	2 (20%)
Papilla, mineralization	1 (10%)		1 (10%)		1 (10%)	
Renal tubule, vacuolization cytoplasmic	9 (90%)	9 (90%)	2 (20%)	1 (10%)		
Urinary bladder	(10)					(10)
Infiltration cellular, mononuclear cell						1 (10%)
Inflammation	1 (10%)					

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Moribund						2
Natural deaths						8
Survivors						
Terminal sacrifice	10	10	10	10	10	
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Esophagus	(10)				(10)	(10)
Muscularis, degeneration					1 (10%)	
Gallbladder	(10)				(10)	(10)
Inflammation						1 (10%)
Intestine large, colon	(10)				(10)	(10)
Inflammation						1 (10%)
Intestine large, cecum	(10)				(10)	(10)
Inflammation						1 (10%)
Goblet cell, hyperplasia					1 (10%)	
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Basophilic focus					1 (10%)	
Eosinophilic focus					1 (10%)	
Fatty change, diffuse						5 (50%)
Inflammation, chronic active	10 (100%)	9 (90%)	10 (100%)	10 (100%)	10 (100%)	2 (20%)
Mixed cell focus				1 (10%)		
Necrosis						10 (100%)
Pigmentation, hemosiderin	1 (10%)					
Bile duct, hyperplasia				1 (10%)	2 (20%)	
Hepatocyte, degeneration		1 (10%)	3 (30%)	10 (100%)	10 (100%)	
Hepatocyte, hypertrophy			3 (30%)	10 (100%)	10 (100%)	
Oval cell, hyperplasia		1 (10%)	10 (100%)	10 (100%)	10 (100%)	5 (50%)
Mesentery						(2)
Fat, inflammation						1 (50%)
Fat, necrosis						1 (50%)
Salivary glands	(10)				(10)	(10)
Cytoplasmic alteration						10 (100%)
Stomach, forestomach	(10)	(10)	(10)	(9)	(10)	(10)
Hyperplasia, squamous						6 (60%)
Mineralization						6 (60%)
Ulcer						6 (60%)
Epithelium, necrosis						1 (10%)
Stomach, glandular	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy					1 (10%)	
Infiltration cellular, polymorphonuclear					1 (10%)	
Mineralization				1 (10%)	2 (20%)	
Epithelium, glands, degeneration					1 (10%)	5 (50%)
Cardiovascular System						
None						

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Endocrine System						
Adrenal cortex	(10)				(10)	(10)
Subcapsular, hyperplasia	10 (100%)				10 (100%)	6 (60%)
Parathyroid gland	(7)				(8)	(8)
Cyst	1 (14%)					
Thyroid gland	(10)				(10)	(10)
Infiltration cellular, lymphocyte					1 (10%)	
Follicle, cyst					1 (10%)	
General Body System						
None						
Genital System						
Clitoral gland	(10)			(1)	(10)	(10)
Pigmentation, melanin				1 (100%)		
Uterus	(10)				(10)	(10)
Endometrium, hyperplasia, cystic	1 (10%)				1 (10%)	
Hematopoietic System						
Lymph node						(3)
Mediastinal, atrophy						1 (33%)
Mediastinal, infiltration cellular, histiocyte						1 (33%)
Pancreatic, atrophy						1 (33%)
Renal, atrophy						1 (33%)
Lymph node, mandibular	(10)	(9)	(9)	(10)	(9)	(8)
Atrophy						7 (88%)
Lymph node, mesenteric	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy						9 (90%)
Infiltration cellular, polymorphonuclear						1 (10%)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Hematopoietic cell proliferation	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	2 (20%)
Lymphoid follicle, atrophy						10 (100%)
Red pulp, depletion cellular						9 (90%)
Thymus	(10)				(10)	(10)
Necrosis, lymphoid						10 (100%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Respiratory System						
Lung	(10)				(10)	(10)
Congestion						1 (10%)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory epithelium, degeneration				2 (20%)	10 (100%)	10 (100%)
Special Senses System						
None						
Urinary System						
Kidney	(10)	(9)	(9)	(10)	(10)	(10)
Nephropathy	5 (50%)				1 (10%)	1 (10%)
Papilla, mineralization					1 (10%)	
Urinary bladder	(10)				(10)	(10)
Infiltration cellular, mononuclear cell					1 (10%)	

APPENDIX B
GENETIC TOXICOLOGY

TABLE B1
Mutagenicity of Estragole in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+10% hamster S9 Trial 1	+10% hamster S9 Trial 2	+10% rat S9 Trial 1	+10% rat S9 Trial 2
TA100	0	129 ± 1.0	100 ± 5.0	88 ± 4.0	103 ± 11.0	107 ± 13.0	115 ± 6.0
	1	126 ± 10.0	96 ± 4.0				
	3.3	122 ± 2.0	102 ± 16.0	82 ± 10.0	115 ± 4.0	96 ± 5.0	129 ± 9.0
	10	116 ± 6.0	106 ± 6.0	87 ± 1.0	91 ± 4.0	108 ± 6.0	116 ± 10.0
	33	109 ± 10.0	113 ± 10.0	97 ± 10.0	100 ± 5.0	111 ± 7.0	111 ± 5.0
	100	73[c]	73[c]	91 ± 3.0	103 ± 8.0	94 ± 4.0	123 ± 10.0
	200				94 ± 4.0[d]		92 ± 1.0[d]
	220			72 ± 3.0[d]		—[c]	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[e]		1,897 ± 39.0	2,119 ± 68.0	1,209 ± 40.0	1,513 ± 12.0	1,718 ± 34.0	2,497 ± 38.0
TA1535	0	26 ± 3.0	21 ± 3.0	11 ± 1.0	9 ± 3.0	11 ± 2.0	21 ± 1.0
	1	26 ± 2.0	26 ± 3.0				
	3.3	33 ± 3.0	21 ± 3.0	11 ± 1.0	11 ± 1.0	8 ± 1.0	21 ± 4.0
	10	32 ± 3.0	17 ± 2.0	9 ± 0.0	10 ± 1.0	11 ± 1.0	28 ± 2.0
	33	29 ± 1.0	20 ± 1.0	11 ± 1.0	10 ± 2.0	7 ± 0.0	22 ± 2.0
	100	7 ± 2.0[d]	5 ± 2.0[d]	9 ± 1.0	10 ± 2.0	9 ± 1.0	19 ± 1.0
	200				5 ± 2.0[d]		8[c]
	220			—[c]		6[c]	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		1,600 ± 25.0	1,257 ± 38.0	192 ± 9.0	147 ± 21.0	203 ± 16.0	196 ± 11.0
TA1537	0	7 ± 2.0	5 ± 2.0	8 ± 2.0	7 ± 2.0	7 ± 2.0	6 ± 1.0
	1	5 ± 2.0	2 ± 1.0				
	3.3	7 ± 1.0	6 ± 1.0	6 ± 1.0	5 ± 1.0	8 ± 2.0	6 ± 2.0
	10	6 ± 3.0	6 ± 0.0	5 ± 2.0	7 ± 0.0	7 ± 2.0	6 ± 1.0
	33	6 ± 1.0	3 ± 1.0	9 ± 2.0	5 ± 1.0	6 ± 1.0	7 ± 3.0
	100	5 ± 0.0[d]	6 ± 1.0[d]	7 ± 2.0	12 ± 2.0	7 ± 1.0	5 ± 1.0
	200				5 ± 3.0[d]		4 ± 0.0[d]
	220			5[c]		—[c]	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		443 ± 29.0	370 ± 36.0	71 ± 8.0	96 ± 9.0	185 ± 9.0	178 ± 9.0
TA98	0	16 ± 2.0	11 ± 2.0	29 ± 3.0	29 ± 3.0	35 ± 4.0	23 ± 2.0
	1	9 ± 3.0	13 ± 1.0				
	3.3	16 ± 1.0	12 ± 5.0	27 ± 3.0	24 ± 1.0	22 ± 2.0	27 ± 1.0
	10	15 ± 2.0	14 ± 1.0	32 ± 2.0	28 ± 2.0	29 ± 1.0	26 ± 2.0
	33	18 ± 1.0	13 ± 2.0	29 ± 3.0	24 ± 4.0	29 ± 3.0	25 ± 1.0
	100	11[c]	11 ± 0.0[d]	19 ± 2.0	20 ± 2.0	25 ± 1.0	26 ± 4.0
	200				18 ± 3.0[d]		12[c]
	220			22[c]		22[c]	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		1,332 ± 61.0	1,237 ± 11.0	1,012 ± 63.0	1,055 ± 12.0	1,579 ± 31.0	1,705 ± 89.0

[a] Studies were performed at EG&G Mason Research Institute. The detailed protocol and these data are presented by Zeiger *et al.* (1987). 0 µg/plate was the solvent control.

[b] Revertants are presented as mean ± standard error from three plates.

[c] Toxic

[d] Slight toxicity

[e] The positive controls in the the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537), and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE B2
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice Following Treatment with Estragole by Gavage for 3 Months[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)
Male					
Corn oil[d]	0	5	1.00 ± 0.22		2.5 ± 0.3
Estragole	37.5	5	1.20 ± 0.34	0.3348	2.6 ± 0.1
	75	5	1.10 ± 0.19	0.4136	2.3 ± 0.3
	150	5	1.20 ± 0.25	0.3348	2.3 ± 0.1
	300	5	1.50 ± 0.16	0.1585	2.0 ± 0.2
	600	5	0.70 ± 0.46	0.7667	2.1 ± 0.2
			P=0.763[e]		
Female					
Corn oil	0	5	1.00 ± 0.22		2.4 ± 0.3
Estragole	37.5	5	0.90 ± 0.46	0.5908	1.8 ± 0.2
	75	5	0.40 ± 0.29	0.9457	2.4 ± 0.1
	150	5	0.90 ± 0.19	0.5908	1.8 ± 0.1
	300	5	0.50 ± 0.27	0.9017	2.3 ± 0.1
			P=0.852		

[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by MacGregor *et al.* (1990). NCE=normochromatic erythrocyte, PCE=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the vehicle control; dosed group values are significant at P≤0.005 for male mice or P≤0.006 for female mice.
[d] Vehicle control
[e] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test, significant at P≤0.025

APPENDIX C
CLINICAL PATHOLOGY RESULTS

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
Hematology						
n						
Day 4	10	10	10	10	10	10
Day 23	10	9	10	10	10	10
Week 14	10	10	10	10	9	10
Hematocrit (%)						
Day 4	40.7 ± 0.7	40.2 ± 0.5	40.1 ± 0.4	40.8 ± 0.5	40.0 ± 0.3	41.9 ± 0.6
Day 23	45.5 ± 0.4	44.8 ± 0.5	45.3 ± 0.4	45.7 ± 0.2	44.4 ± 0.3	35.8 ± 1.3**
Week 14	45.1 ± 0.4	43.6 ± 0.3	44.3 ± 0.3	44.6 ± 0.2	42.7 ± 0.4**	33.5 ± 1.0**
Hemoglobin (g/dL)						
Day 4	13.2 ± 0.2	13.1 ± 0.2	13.1 ± 0.1	13.2 ± 0.1	13.1 ± 0.1	13.9 ± 0.3
Day 23	15.2 ± 0.2	14.7 ± 0.2	15.1 ± 0.2	15.0 ± 0.1	14.8 ± 0.1	11.6 ± 0.5**
Week 14	15.0 ± 0.2	14.7 ± 0.1	14.7 ± 0.1	14.9 ± 0.1	13.9 ± 0.1**	11.1 ± 0.3**
Erythrocytes (10^6/µL)						
Day 4	7.03 ± 0.15	6.94 ± 0.09	7.00 ± 0.09	7.08 ± 0.09	7.06 ± 0.05	7.56 ± 0.13**
Day 23	8.05 ± 0.09	7.96 ± 0.08	8.14 ± 0.09	8.13 ± 0.06	8.06 ± 0.06	7.64 ± 0.15
Week 14	8.73 ± 0.11	8.49 ± 0.05	8.70 ± 0.07	8.75 ± 0.05	8.72 ± 0.07	9.45 ± 0.10**
Reticulocytes (10^6/µL)						
Day 4	0.50 ± 0.02	0.48 ± 0.03	0.44 ± 0.02	0.48 ± 0.03	0.38 ± 0.03**	0.22 ± 0.01**
Day 23	0.22 ± 0.02	0.16 ± 0.02	0.18 ± 0.02	0.19 ± 0.02	0.16 ± 0.02	0.28 ± 0.03
Week 14	0.13 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.13 ± 0.01
Nucleated erythrocytes (10^3/µL)						
Day 4	0.14 ± 0.04	0.19 ± 0.04	0.18 ± 0.05	0.16 ± 0.04	0.19 ± 0.05	0.02 ± 0.01
Day 23	0.13 ± 0.05	0.17 ± 0.05	0.05 ± 0.02	0.03 ± 0.02	0.09 ± 0.05	0.50 ± 0.12
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[b]
Mean cell volume (fL)						
Day 4	57.9 ± 0.3	57.9 ± 0.1	57.2 ± 0.2	57.6 ± 0.3	56.7 ± 0.4*	55.5 ± 0.3**
Day 23	56.6 ± 0.3	56.3 ± 0.2	55.7 ± 0.2	56.3 ± 0.2	55.1 ± 0.2**	46.7 ± 0.9**
Week 14	51.7 ± 0.2	51.4 ± 0.2	51.0 ± 0.2*	51.0 ± 0.2*	48.9 ± 0.2**	35.6 ± 1.3**
Mean cell hemoglobin (pg)						
Day 4	18.8 ± 0.1	18.9 ± 0.1	18.8 ± 0.1	18.7 ± 0.1	18.5 ± 0.1	18.4 ± 0.1*
Day 23	18.8 ± 0.1	18.4 ± 0.1*	18.5 ± 0.1*	18.5 ± 0.1*	18.4 ± 0.1**	15.1 ± 0.3**
Week 14	17.2 ± 0.1	17.3 ± 0.1	16.9 ± 0.1	17.1 ± 0.1	16.0 ± 0.1**	11.8 ± 0.4**
Mean cell hemoglobin concentration (g/dL)						
Day 4	32.5 ± 0.1	32.6 ± 0.2	32.8 ± 0.1	32.5 ± 0.2	32.7 ± 0.1	33.1 ± 0.2*
Day 23	33.3 ± 0.2	32.7 ± 0.2	33.2 ± 0.2	32.8 ± 0.2	33.3 ± 0.2	32.2 ± 0.2**
Week 14	33.2 ± 0.1	33.7 ± 0.2	33.2 ± 0.1	33.4 ± 0.1	32.6 ± 0.1	33.2 ± 0.2
Platelets (10^3/µL)						
Day 4	983.9 ± 10.7	1,021.1 ± 12.6	973.2 ± 27.6	986.2 ± 22.2	992.8 ± 21.9	1,021.8 ± 20.9
Day 23	886.2 ± 16.6	957.3 ± 13.2**	973.2 ± 25.7**	1,042.7 ± 22.2**	1,133.1 ± 33.0**	1,896.3 ± 58.1**
Week 14	690.7 ± 10.2	755.6 ± 15.4**	777.5 ± 26.3**	912.8 ± 20.2**	1,090.4 ± 30.7**	1,780.3 ± 113.0**
Leukocytes (10^3/µL)						
Day 4	8.83 ± 0.31	9.06 ± 0.37	9.26 ± 0.32	9.85 ± 0.39	9.15 ± 0.36	7.16 ± 0.34
Day 23	12.52 ± 0.46	12.63 ± 0.48	13.75 ± 0.62	12.35 ± 0.81	12.54 ± 0.54	12.48 ± 0.81
Week 14	6.27 ± 0.35	8.07 ± 0.41**	8.14 ± 0.77*	9.50 ± 0.54**	10.17 ± 0.59**	14.89 ± 1.06**
Segmented neutrophils (10^3/µL)						
Day 4	1.15 ± 0.09	1.49 ± 0.13	1.24 ± 0.20	1.28 ± 0.16	1.33 ± 0.16	1.40 ± 0.11
Day 23	1.27 ± 0.18	1.33 ± 0.15	1.04 ± 0.14	0.96 ± 0.16	1.41 ± 0.17	1.78 ± 0.23
Week 14	0.98 ± 0.08	1.23 ± 0.15	1.25 ± 0.14	1.18 ± 0.11	1.38 ± 0.15*	2.12 ± 0.21**[b]

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male (continued)						
Hematology (continued)						
n						
Day 4	10	10	10	10	10	10
Day 23	10	9	10	10	10	10
Week 14	10	10	10	10	9	10
Bands (10^3/µL)						
Day 4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[b]
Lymphocytes (10^3/µL)						
Day 4	7.61 ± 0.26	7.44 ± 0.32	7.91 ± 0.36	8.51 ± 0.41	7.70 ± 0.45	5.68 ± 0.26*
Day 23	10.48 ± 0.35	10.67 ± 0.41	11.82 ± 0.62	10.65 ± 0.69	10.33 ± 0.46	10.05 ± 0.75
Week 14	5.01 ± 0.33	6.61 ± 0.27**	6.57 ± 0.64*	7.97 ± 0.59**	8.36 ± 0.49**	12.12 ± 0.95**[b]
Monocytes (10^3/µL)						
Day 4	0.04 ± 0.02	0.08 ± 0.02	0.06 ± 0.02	0.05 ± 0.02	0.08 ± 0.04	0.06 ± 0.02
Day 23	0.70 ± 0.07	0.52 ± 0.05	0.86 ± 0.10	0.66 ± 0.10	0.70 ± 0.08	0.63 ± 0.10
Week 14	0.19 ± 0.04	0.17 ± 0.04	0.24 ± 0.06	0.26 ± 0.05	0.37 ± 0.08	0.32 ± 0.09[b]
Basophils (10^3/µL)						
Day 4	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 23	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000[b]
Eosinophils (10^3/µL)						
Day 4	0.04 ± 0.02	0.04 ± 0.02	0.05 ± 0.02	0.02 ± 0.01	0.04 ± 0.02	0.02 ± 0.01
Day 23	0.08 ± 0.03	0.11 ± 0.05	0.03 ± 0.02	0.08 ± 0.03	0.10 ± 0.03	0.02 ± 0.02
Week 14	0.09 ± 0.02	0.06 ± 0.02	0.08 ± 0.03	0.09 ± 0.04	0.06 ± 0.03	0.07 ± 0.03[b]
Clinical Chemistry						
n	10	10	10	10	10	10
Urea nitrogen (mg/dL)						
Day 4	9.5 ± 0.4	11.0 ± 0.5	9.4 ± 0.4	9.9 ± 0.6	9.3 ± 0.3	8.1 ± 0.4*
Day 23	12.4 ± 0.7	12.1 ± 0.7	11.3 ± 0.4	12.0 ± 0.3	10.8 ± 0.5	11.7 ± 0.4
Week 14	12.2 ± 0.6	10.6 ± 0.5	11.3 ± 0.6	12.0 ± 0.4	10.3 ± 0.5	10.8 ± 0.2
Creatinine (mg/dL)						
Day 4	0.40 ± 0.00	0.43 ± 0.02	0.40 ± 0.00	0.42 ± 0.01	0.42 ± 0.02[b]	0.41 ± 0.01
Day 23	0.54 ± 0.02	0.50 ± 0.00	0.50 ± 0.00	0.53 ± 0.02	0.53 ± 0.02	0.50 ± 0.00
Week 14	0.67 ± 0.02	0.66 ± 0.02	0.66 ± 0.02	0.69 ± 0.02	0.64 ± 0.02	0.60 ± 0.02*
Total protein (g/dL)						
Day 4	5.7 ± 0.1	5.6 ± 0.1	5.5 ± 0.0	5.4 ± 0.1*	5.1 ± 0.1**	4.4 ± 0.1**
Day 23	6.6 ± 0.1	6.3 ± 0.1*	6.3 ± 0.1*	6.3 ± 0.1*	5.7 ± 0.1**	5.6 ± 0.1**
Week 14	6.9 ± 0.1	6.7 ± 0.1	6.5 ± 0.1	6.2 ± 0.1**	7.1 ± 0.1	7.6 ± 0.1
Albumin (g/dL)						
Day 4	4.0 ± 0.0	3.9 ± 0.1	3.9 ± 0.0	3.8 ± 0.0**	3.6 ± 0.0**	3.1 ± 0.1**
Day 23	4.4 ± 0.0	4.3 ± 0.0*	4.3 ± 0.1*	4.3 ± 0.0*	4.0 ± 0.0**	3.8 ± 0.1**
Week 14	4.5 ± 0.0	4.4 ± 0.0	4.4 ± 0.0	4.2 ± 0.0**	4.5 ± 0.1	4.6 ± 0.1
Alanine aminotransferase (IU/L)						
Day 4	68 ± 1	74 ± 2	71 ± 3	79 ± 2**	84 ± 2**	119 ± 8**
Day 23	55 ± 2	53 ± 2	53 ± 2	59 ± 2	78 ± 4**	92 ± 3**
Week 14	63 ± 6	52 ± 1	51 ± 1	61 ± 3	77 ± 6*	182 ± 20**

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male (continued)						
Clinical Chemistry (continued)						
n	10	10	10	10	10	10
Alkaline phosphatase (IU/L)						
Day 4	885 ± 24	962 ± 19	895 ± 23	923 ± 16	887 ± 22	913 ± 34
Day 23	667 ± 13	638 ± 12	622 ± 19	668 ± 17	656 ± 19	673 ± 20
Week 14	261 ± 7	243 ± 3	251 ± 5	269 ± 5	277 ± 8	303 ± 9**
Creatine kinase (IU/L)						
Day 4	388 ± 43	403 ± 32	372 ± 49[b]	426 ± 29	365 ± 47[b]	528 ± 62
Day 23	775 ± 162	670 ± 74	943 ± 378	665 ± 66	700 ± 122	376 ± 30*
Week 14	279 ± 71	168 ± 27	407 ± 78	388 ± 94	203 ± 44[b]	384 ± 80
Sorbitol dehydrogenase (IU/L)						
Day 4	18 ± 1	20 ± 0	17 ± 1	18 ± 0	20 ± 1	24 ± 1**
Day 23	24 ± 2	22 ± 1	23 ± 1	25 ± 1	31 ± 3	28 ± 1
Week 14	19 ± 2	14 ± 1	19 ± 1	22 ± 1	23 ± 2	34 ± 2**
Bile salts (µmol/L)						
Day 4	20.5 ± 1.8	23.0 ± 2.7	30.3 ± 2.5**	32.1 ± 2.9**	39.3 ± 3.2**	68.8 ± 12.1**
Day 23	28.5 ± 5.3	20.4 ± 2.1	32.8 ± 2.3	41.4 ± 4.8*	52.2 ± 5.3**	60.2 ± 6.7**
Week 14	15.2 ± 1.4	20.3 ± 1.8*	25.3 ± 2.9**	36.4 ± 4.1**	53.1 ± 6.4**	129.0 ± 11.5**
Iron (µg/dL)						
Week 14	200.1 ± 8.6	179.3 ± 4.5	164.9 ± 5.3**	184.6 ± 5.0	189.1 ± 6.6	161.3 ± 9.2*
Unsaturated iron binding capacity (µg/dL)						
Week 14	456.4 ± 16.7	480.4 ± 13.1	490.0 ± 14.2	468.3 ± 6.5	845.0 ± 25.2**	1,417.5 ± 45.8**
Total iron binding capacity (µg/dL)						
Week 14	656.5 ± 12.9	659.7 ± 14.9	654.9 ± 11.8	652.9 ± 6.3	1,034.1 ± 21.7**	1,578.8 ± 43.8**
Female						
Hematology						
n	10	10	10	10	10	10
Hematocrit (%)						
Day 4	42.4 ± 0.7	44.3 ± 0.6	42.8 ± 0.8	43.8 ± 0.8	42.2 ± 0.9	43.1 ± 0.6
Day 23	47.1 ± 0.5	46.5 ± 0.3	47.1 ± 0.6	47.8 ± 0.5	46.8 ± 0.4	42.4 ± 0.5**
Week 14	45.7 ± 0.5	45.4 ± 0.2	45.1 ± 0.5	44.5 ± 0.4	45.6 ± 0.2	41.0 ± 0.4**
Hemoglobin (g/dL)						
Day 4	14.0 ± 0.2	14.6 ± 0.2	14.2 ± 0.2	14.5 ± 0.3	13.8 ± 0.3	14.3 ± 0.2
Day 23	15.6 ± 0.2	15.3 ± 0.1	15.6 ± 0.2	15.7 ± 0.2	15.4 ± 0.1	13.8 ± 0.2**
Week 14	15.2 ± 0.1	15.1 ± 0.1	14.9 ± 0.1	14.7 ± 0.2	15.0 ± 0.1	13.2 ± 0.1**
Erythrocytes (10^6/µL)						
Day 4	7.36 ± 0.12	7.75 ± 0.11	7.50 ± 0.15	7.69 ± 0.15	7.44 ± 0.18	7.74 ± 0.11
Day 23	8.32 ± 0.10	8.29 ± 0.06	8.37 ± 0.11	8.46 ± 0.11	8.41 ± 0.07	8.64 ± 0.09
Week 14	8.39 ± 0.09	8.34 ± 0.05	8.27 ± 0.09	8.16 ± 0.08	8.55 ± 0.04	9.21 ± 0.10**
Reticulocytes (10^6/µL)						
Day 4	0.35 ± 0.04	0.31 ± 0.03	0.39 ± 0.02	0.32 ± 0.02	0.33 ± 0.02	0.25 ± 0.01
Day 23	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.11 ± 0.02
Week 14	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.07 ± 0.00	0.08 ± 0.01
Nucleated erythrocytes (10^3/µL)						
Day 4	0.09 ± 0.03	0.08 ± 0.03	0.16 ± 0.03	0.06 ± 0.02	0.06 ± 0.03	0.00 ± 0.00*
Day 23	0.02 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.02
Week 14	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Female (continued)						
Hematology (continued)						
n	10	10	10	10	10	10
Mean cell volume (fL)						
Day 4	57.6 ± 0.2	57.1 ± 0.2	57.1 ± 0.3	57.0 ± 0.3	56.7 ± 0.3*	55.6 ± 0.2**
Day 23	56.7 ± 0.1	56.0 ± 0.1**	56.3 ± 0.2*	56.4 ± 0.3*	55.7 ± 0.2**	49.1 ± 0.6**
Week 14	54.5 ± 0.1	54.5 ± 0.1	54.5 ± 0.1	54.5 ± 0.2	53.3 ± 0.1**	44.6 ± 0.7**
Mean cell hemoglobin (pg)						
Day 4	19.0 ± 0.1	18.8 ± 0.1	18.9 ± 0.1	18.8 ± 0.1	18.6 ± 0.1*	18.5 ± 0.0**
Day 23	18.8 ± 0.1	18.5 ± 0.1**	18.6 ± 0.1*	18.6 ± 0.1*	18.4 ± 0.1**	16.0 ± 0.2**
Week 14	18.1 ± 0.1	18.0 ± 0.1	18.0 ± 0.1	18.0 ± 0.1	17.6 ± 0.1**	14.3 ± 0.2**
Mean cell hemoglobin concentration (g/dL)						
Day 4	33.0 ± 0.2	33.0 ± 0.2	33.1 ± 0.2	33.0 ± 0.1	32.9 ± 0.1	33.2 ± 0.1
Day 23	33.2 ± 0.1	33.0 ± 0.2	33.1 ± 0.2	33.0 ± 0.2	33.0 ± 0.1	32.6 ± 0.1**
Week 14	33.3 ± 0.1	33.2 ± 0.1	33.0 ± 0.1	33.0 ± 0.1	33.0 ± 0.1	32.2 ± 0.1**
Platelets (10^3/µL)						
Day 4	881.9 ± 56.8	914.0 ± 46.4	849.8 ± 33.7	961.4 ± 23.7	880.0 ± 14.2	970.2 ± 21.0
Day 23	861.4 ± 15.2	835.5 ± 39.1	859.9 ± 31.1	875.7 ± 35.4	914.7 ± 24.2	1,410.3 ± 45.8**
Week 14	728.2 ± 16.7	763.2 ± 11.9	801.3 ± 16.4**	824.9 ± 10.8**	857.8 ± 20.3**	1,048.1 ± 31.2**
Leukocytes (10^3/µL)						
Day 4	9.11 ± 0.48	10.11 ± 0.35	10.19 ± 0.65	9.71 ± 0.52	10.07 ± 0.31	8.95 ± 0.19
Day 23	11.02 ± 0.52	12.38 ± 0.73	12.29 ± 0.85	11.47 ± 1.12	13.10 ± 0.62	11.34 ± 1.00
Week 14	6.77 ± 0.27	8.01 ± 0.35*	8.24 ± 0.48*	10.57 ± 0.31**	8.65 ± 0.60**	10.95 ± 0.56**
Segmented neutrophils (10^3/µL)						
Day 4	1.02 ± 0.11	1.08 ± 0.08	1.04 ± 0.08	1.12 ± 0.12	1.08 ± 0.09	1.24 ± 0.07
Day 23	0.97 ± 0.08	1.21 ± 0.09	0.89 ± 0.13	1.05 ± 0.10	1.27 ± 0.13	1.26 ± 0.20
Week 14	0.98 ± 0.07	0.80 ± 0.04	0.92 ± 0.08	1.04 ± 0.06	1.07 ± 0.11	1.23 ± 0.14
Bands (10^3/µL)						
Day 4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/µL)						
Day 4	7.74 ± 0.39	8.59 ± 0.35	8.75 ± 0.62	8.18 ± 0.37	8.61 ± 0.26	7.26 ± 0.19
Day 23	9.63 ± 0.47	10.70 ± 0.73	10.98 ± 0.76	10.01 ± 1.02	11.42 ± 0.61	9.74 ± 0.76
Week 14	5.66 ± 0.27	7.02 ± 0.34*	7.15 ± 0.45*	9.32 ± 0.30**	7.31 ± 0.57**	9.57 ± 0.63**
Monocytes (10^3/µL)						
Day 4	0.32 ± 0.06	0.41 ± 0.03	0.39 ± 0.08	0.36 ± 0.07	0.36 ± 0.05	0.40 ± 0.08
Day 23	0.39 ± 0.06	0.42 ± 0.05	0.39 ± 0.07	0.35 ± 0.05	0.37 ± 0.05	0.28 ± 0.08
Week 14	0.08 ± 0.02	0.11 ± 0.02	0.12 ± 0.04	0.17 ± 0.04*	0.19 ± 0.02**	0.14 ± 0.03*
Basophils (10^3/µL)						
Day 4	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 23	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/µL)						
Day 4	0.04 ± 0.02	0.04 ± 0.02	0.01 ± 0.01	0.05 ± 0.03	0.02 ± 0.01	0.05 ± 0.03
Day 23	0.03 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.06 ± 0.02	0.04 ± 0.02	0.07 ± 0.03
Week 14	0.06 ± 0.02	0.07 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.08 ± 0.03	0.02 ± 0.01

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Estragole

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Female (continued)						
Clinical Chemistry						
n	10	10	10	10	10	10
Urea nitrogen (mg/dL)						
Day 4	10.8 ± 0.7	12.8 ± 0.7	10.5 ± 0.6	11.4 ± 0.7	10.4 ± 0.5	9.1 ± 0.2[b]
Day 23	15.2 ± 0.7	13.5 ± 0.4*	13.0 ± 0.5*	12.3 ± 0.5**	11.2 ± 0.5**	10.5 ± 0.4**
Week 14	13.1 ± 0.4	14.3 ± 0.4	12.3 ± 0.4	13.5 ± 0.7	12.7 ± 0.4	12.8 ± 0.6
Creatinine (mg/dL)						
Day 4	0.48 ± 0.01	0.46 ± 0.02	0.47 ± 0.02	0.47 ± 0.02	0.43 ± 0.02	0.43 ± 0.02
Day 23	0.51 ± 0.01	0.53 ± 0.02	0.55 ± 0.02	0.54 ± 0.02	0.51 ± 0.02	0.49 ± 0.01
Week 14	0.57 ± 0.02	0.57 ± 0.02	0.57 ± 0.02	0.56 ± 0.02	0.59 ± 0.01	0.57 ± 0.02
Total protein (g/dL)						
Day 4	6.0 ± 0.1	6.1 ± 0.1	5.8 ± 0.1	5.8 ± 0.1	5.5 ± 0.1**	4.9 ± 0.1**[b]
Day 23	6.5 ± 0.1	6.1 ± 0.1*	6.0 ± 0.1**	6.0 ± 0.1**	5.5 ± 0.1**	5.1 ± 0.1**
Week 14	6.7 ± 0.1	6.5 ± 0.1	6.3 ± 0.1	6.0 ± 0.1**	6.1 ± 0.0*	7.4 ± 0.1
Albumin (g/dL)						
Day 4	4.2 ± 0.1	4.4 ± 0.0	4.1 ± 0.1	4.1 ± 0.0	3.9 ± 0.0**	3.5 ± 0.0**[b]
Day 23	4.6 ± 0.0	4.4 ± 0.1*	4.3 ± 0.0**	4.3 ± 0.0**	4.1 ± 0.0**	3.7 ± 0.0**
Week 14	4.7 ± 0.0	4.7 ± 0.1	4.6 ± 0.1	4.3 ± 0.0**	4.4 ± 0.0**	5.0 ± 0.0
Alanine aminotransferase (IU/L)						
Day 4	60 ± 2	61 ± 3	63 ± 1	63 ± 2	68 ± 2**	109 ± 4**
Day 23	48 ± 2	49 ± 1	49 ± 2	53 ± 1*	61 ± 2**	93 ± 4**
Week 14	49 ± 2	40 ± 2	40 ± 1	45 ± 1	51 ± 1	97 ± 6**
Alkaline phosphatase (IU/L)						
Day 4	790 ± 19	761 ± 16	762 ± 17	743 ± 14	726 ± 10*	765 ± 13
Day 23	497 ± 11	494 ± 13	512 ± 10	517 ± 7	486 ± 13	624 ± 19**
Week 14	222 ± 6	236 ± 10	219 ± 7	229 ± 11	221 ± 6	226 ± 10
Creatine kinase (IU/L)						
Day 4	423 ± 57	329 ± 43	479 ± 93	318 ± 42	365 ± 46	409 ± 48[b]
Day 23	447 ± 44	463 ± 40[b]	497 ± 71[b]	584 ± 122	626 ± 68	592 ± 53
Week 14	290 ± 42	270 ± 46	177 ± 31	209 ± 42	391 ± 62	246 ± 52[b]
Sorbitol dehydrogenase (IU/L)						
Day 4	18 ± 1	17 ± 1	16 ± 1	18 ± 1	18 ± 0	22 ± 2*
Day 23	18 ± 1	18 ± 1	19 ± 1	18 ± 0	18 ± 1	21 ± 2
Week 14	16 ± 1	17 ± 1	16 ± 1	16 ± 1	23 ± 1**	34 ± 2**
Bile salts (µmol/L)						
Day 4	22.7 ± 3.3	18.1 ± 2.3	19.6 ± 1.4	22.4 ± 1.6	23.1 ± 2.3	45.1 ± 2.8**
Day 23	19.2 ± 2.8	23.6 ± 3.0	31.7 ± 3.7	27.0 ± 3.9	33.7 ± 2.7**	73.7 ± 9.9**
Week 14	18.8 ± 1.8	17.9 ± 1.3	25.9 ± 2.2*	24.5 ± 2.5*	32.7 ± 2.2**	53.1 ± 3.8**
Iron (µg/dL)						
Week 14	321.8 ± 9.2	320.1 ± 13.6	308.1 ± 10.9	328.9 ± 29.6	247.1 ± 14.1**	267.3 ± 20.0**
Unsaturated iron binding capacity (µg/dL)						
Week 14	275.5 ± 11.9	286.0 ± 17.4	296.7 ± 15.9	262.5 ± 23.8	360.0 ± 13.8**	884.0 ± 24.6**
Total iron binding capacity (µg/dL)						
Week 14	597.3 ± 8.7	606.1 ± 7.5	604.8 ± 13.2	591.4 ± 10.1	607.1 ± 9.0	1,151.3 ± 34.1**

* Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test
** P≤0.01
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9

TABLE C2
Hematology Data for Mice in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
n	10	9	10	10	10	9
Hematocrit (%)	49.9 ± 0.3	49.7 ± 0.4	51.1 ± 0.7	50.7 ± 0.4	51.2 ± 0.5	49.6 ± 0.6
Hemoglobin (g/dL)	16.3 ± 0.1	16.3 ± 0.2	16.7 ± 0.3	16.6 ± 0.1	16.8 ± 0.2	16.2 ± 0.2
Erythrocytes (10^6/µL)	10.76 ± 0.07	10.69 ± 0.12	11.08 ± 0.18	10.94 ± 0.11	10.94 ± 0.12	10.44 ± 0.13
Reticulocytes (10^6/µL)	0.07 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.04 ± 0.01
Nucleated erythrocytes (10^3/µL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	46.3 ± 0.2	46.5 ± 0.2	46.2 ± 0.2	46.4 ± 0.1	46.8 ± 0.1*	47.4 ± 0.1**
Mean cell hemoglobin (pg)	15.1 ± 0.1	15.2 ± 0.1	15.1 ± 0.0	15.2 ± 0.1	15.3 ± 0.1**	15.5 ± 0.0**
Mean cell hemoglobin concentration (g/dL)	32.6 ± 0.1	32.8 ± 0.1	32.7 ± 0.1	32.7 ± 0.1	32.8 ± 0.1	32.7 ± 0.1
Platelets (10^3/µL)	660.9 ± 26.1	685.6 ± 19.7	684.7 ± 42.8	641.6 ± 30.9	659.0 ± 23.5	784.3 ± 24.5*
Leukocytes (10^3/µL)	2.76 ± 0.38	2.73 ± 0.55	3.92 ± 0.43	1.96 ± 0.37	3.83 ± 0.41	5.47 ± 0.47**
Segmented neutrophils (10^3/µL)	0.33 ± 0.06	0.44 ± 0.12	0.44 ± 0.06	0.32 ± 0.08	0.46 ± 0.04	0.75 ± 0.05**
Bands (10^3/µL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/µL)	2.39 ± 0.32	2.24 ± 0.42	3.41 ± 0.39	1.60 ± 0.29	3.31 ± 0.37	4.67 ± 0.47**
Monocytes (10^3/µL)	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Basophils (10^3/µL)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/µL)	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.05 ± 0.02	0.04 ± 0.01
Female						
n	10	10	10	10	9	0
Hematocrit (%)	47.6 ± 0.5	48.6 ± 0.4	48.4 ± 0.7	48.8 ± 0.4	47.6 ± 0.5	
Hemoglobin (g/dL)	15.6 ± 0.2	16.0 ± 0.1	16.0 ± 0.3	15.9 ± 0.1	15.4 ± 0.2	
Erythrocytes (10^6/µL)	10.13 ± 0.11	10.24 ± 0.08	10.14 ± 0.15	10.13 ± 0.10	9.82 ± 0.12	
Reticulocytes (10^6/µL)	0.10 ± 0.01	0.10 ± 0.01	0.14 ± 0.01	0.10 ± 0.01	0.13 ± 0.01	
Nucleated erythrocytes (10^3/µL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
Mean cell volume (fL)	47.0 ± 0.1	47.4 ± 0.1	47.8 ± 0.2**	48.2 ± 0.2**	48.5 ± 0.2**	
Mean cell hemoglobin (pg)	15.4 ± 0.1	15.6 ± 0.1	15.8 ± 0.1**	15.7 ± 0.1**	15.7 ± 0.1**	
Mean cell hemoglobin concentration (g/dL)	32.8 ± 0.1	32.9 ± 0.1	33.0 ± 0.1	32.6 ± 0.1	32.4 ± 0.1*	
Platelets (10^3/µL)	786.7 ± 31.3	729.3 ± 30.5	695.8 ± 37.1	795.9 ± 41.3	920.9 ± 28.2	
Leukocytes (10^3/µL)	2.81 ± 0.07	3.27 ± 0.27	2.82 ± 0.26	3.98 ± 0.27*	5.26 ± 0.25**	
Segmented neutrophils (10^3/µL)	0.26 ± 0.03	0.36 ± 0.05	0.41 ± 0.05*	0.51 ± 0.02**	0.62 ± 0.10**	
Bands (10^3/µL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
Lymphocytes (10^3/µL)	2.50 ± 0.05	2.86 ± 0.22	2.38 ± 0.22	3.41 ± 0.26*	4.58 ± 0.25**	
Monocytes (10^3/µL)	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	
Basophils (10^3/µL)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	
Eosinophils (10^3/µL)	0.04 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.04 ± 0.01	0.03 ± 0.02	

* Significantly different (P≤0.05) from the vehicle control group by Dunn's or Shirley's test
** P≤0.01
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.

APPENDIX D
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE D1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
n	10	10	10	10	10	10
Male						
Necropsy body wt	338 ± 5	348 ± 5	342 ± 4	328 ± 5	285 ± 5**	242 ± 4**
Heart						
Absolute	0.999 ± 0.028	1.014 ± 0.017	1.022 ± 0.019	1.012 ± 0.013	0.922 ± 0.027	0.935 ± 0.036
Relative	2.961 ± 0.074	2.917 ± 0.041	2.989 ± 0.036	3.088 ± 0.061	3.233 ± 0.055*	3.856 ± 0.127**
R. Kidney						
Absolute	0.993 ± 0.027	1.122 ± 0.016**	1.094 ± 0.031*	1.073 ± 0.023	1.017 ± 0.030	0.987 ± 0.019
Relative	2.943 ± 0.074	3.230 ± 0.047**	3.198 ± 0.070**	3.270 ± 0.061**	3.565 ± 0.054**	4.073 ± 0.065**
Liver						
Absolute	13.475 ± 0.267	14.494 ± 0.247	13.876 ± 0.337	14.321 ± 0.346	18.920 ± 0.821**	27.226 ± 0.900**
Relative	39.952 ± 0.768	41.716 ± 0.733	40.568 ± 0.579	43.609 ± 0.702	66.204 ± 1.875**	112.264 ± 3.042**
Lung						
Absolute	1.494 ± 0.045	1.720 ± 0.042	1.708 ± 0.044	1.672 ± 0.078	1.493 ± 0.040	1.286 ± 0.052**
Relative	4.428 ± 0.130	4.954 ± 0.133*	5.001 ± 0.127*	5.110 ± 0.267*	5.265 ± 0.196**	5.308 ± 0.197**
R. Testis						
Absolute	1.338 ± 0.027	1.413 ± 0.022	1.366 ± 0.021	1.458 ± 0.060	0.949 ± 0.039**	0.554 ± 0.015**
Relative	3.970 ± 0.090	4.067 ± 0.071	3.997 ± 0.036	4.443 ± 0.177	3.337 ± 0.139**	2.289 ± 0.058**
Thymus						
Absolute	0.319 ± 0.013	0.323 ± 0.018	0.350 ± 0.010	0.323 ± 0.011	0.269 ± 0.008*	0.278 ± 0.015*
Relative	0.944 ± 0.030	0.930 ± 0.051	1.025 ± 0.034	0.986 ± 0.038	0.947 ± 0.031	1.148 ± 0.065**
Female						
Necropsy body wt	196 ± 3	199 ± 4	197 ± 3	192 ± 2	179 ± 3**	169 ± 3**
Heart						
Absolute	0.672 ± 0.013	0.651 ± 0.018	0.678 ± 0.017	0.662 ± 0.015	0.622 ± 0.012	0.692 ± 0.018
Relative	3.439 ± 0.061	3.270 ± 0.064	3.443 ± 0.058	3.456 ± 0.079	3.474 ± 0.063	4.105 ± 0.085**
R. Kidney						
Absolute	0.666 ± 0.014	0.698 ± 0.018	0.719 ± 0.013	0.696 ± 0.012	0.688 ± 0.013	0.709 ± 0.016
Relative	3.407 ± 0.057	3.506 ± 0.054	3.655 ± 0.051**	3.633 ± 0.048**	3.842 ± 0.046**	4.207 ± 0.079**
Liver						
Absolute	6.561 ± 0.206	7.083 ± 0.169	7.488 ± 0.168**	7.913 ± 0.112**	8.607 ± 0.181**	15.420 ± 0.284**
Relative	33.540 ± 0.811	35.622 ± 0.809	38.043 ± 0.520**	41.335 ± 0.574**	48.025 ± 0.539**	91.689 ± 2.036**
Lung						
Absolute	1.146 ± 0.061	1.102 ± 0.042	1.141 ± 0.048	1.114 ± 0.047	1.144 ± 0.052	1.163 ± 0.102
Relative	5.847 ± 0.251	5.543 ± 0.205	5.801 ± 0.234	5.822 ± 0.250	6.390 ± 0.289	6.837 ± 0.480
Thymus						
Absolute	0.256 ± 0.007	0.270 ± 0.015	0.254 ± 0.006	0.249 ± 0.010	0.263 ± 0.008	0.254 ± 0.011
Relative	1.309 ± 0.034	1.359 ± 0.074	1.293 ± 0.028	1.298 ± 0.047	1.469 ± 0.053*	1.505 ± 0.045**

* Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE D2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Gavage Study of Estragole[a]

	Vehicle Control	37.5 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	600 mg/kg
Male						
n	10	10	10	10	10	9
Necropsy body wt	36.6 ± 1.1	37.1 ± 1.0	37.8 ± 1.3	34.8 ± 0.4	31.2 ± 0.8**	28.8 ± 0.4**
Heart						
Absolute	0.185 ± 0.007	0.190 ± 0.009	0.183 ± 0.008	0.198 ± 0.007	0.181 ± 0.009	0.156 ± 0.004*
Relative	5.056 ± 0.121	5.133 ± 0.254	4.842 ± 0.144	5.678 ± 0.174*	5.788 ± 0.209*	5.419 ± 0.104*
R. Kidney						
Absolute	0.279 ± 0.004	0.275 ± 0.005	0.278 ± 0.008	0.265 ± 0.006	0.246 ± 0.007**	0.231 ± 0.006**
Relative	7.676 ± 0.273	7.436 ± 0.156	7.368 ± 0.120	7.602 ± 0.161	7.879 ± 0.136	8.031 ± 0.168
Liver						
Absolute	1.546 ± 0.051	1.639 ± 0.044	1.793 ± 0.077*	1.784 ± 0.049*	1.660 ± 0.060	1.676 ± 0.048
Relative	42.276 ± 0.667	44.194 ± 0.762	47.398 ± 0.731**	51.181 ± 0.964**	53.108 ± 0.768**	58.160 ± 1.167**
Lung						
Absolute	0.316 ± 0.012	0.305 ± 0.012	0.303 ± 0.013	0.338 ± 0.014	0.319 ± 0.012	0.254 ± 0.020*
Relative	8.708 ± 0.439	8.275 ± 0.394	8.068 ± 0.379	9.718 ± 0.425	10.214 ± 0.298	8.773 ± 0.589
R. Testis						
Absolute	0.119 ± 0.002	0.124 ± 0.001	0.123 ± 0.003	0.123 ± 0.002	0.117 ± 0.002	0.115 ± 0.003
Relative	3.272 ± 0.115	3.360 ± 0.082	3.255 ± 0.056	3.520 ± 0.037*	3.763 ± 0.079**	4.010 ± 0.060**
Thymus						
Absolute	0.047 ± 0.002	0.049 ± 0.004	0.055 ± 0.004	0.049 ± 0.004	0.047 ± 0.003	0.039 ± 0.002
Relative	1.293 ± 0.061	1.309 ± 0.095	1.459 ± 0.060	1.415 ± 0.112	1.501 ± 0.084	1.357 ± 0.068
Female						
n	10	10	10	10	10	0
Necropsy body wt	29.4 ± 0.7	27.5 ± 0.8*	26.2 ± 0.8**	24.8 ± 0.2**	24.3 ± 0.3**	
Heart						
Absolute	0.140 ± 0.005	0.135 ± 0.003	0.144 ± 0.005	0.128 ± 0.002	0.127 ± 0.003	
Relative	4.785 ± 0.215	4.927 ± 0.101	5.527 ± 0.178*	5.163 ± 0.088*	5.250 ± 0.108*	
R. Kidney						
Absolute	0.177 ± 0.006	0.170 ± 0.006	0.176 ± 0.004	0.166 ± 0.004	0.167 ± 0.004	
Relative	6.043 ± 0.198	6.186 ± 0.134	6.772 ± 0.214**	6.681 ± 0.154**	6.870 ± 0.158**	
Liver						
Absolute	1.286 ± 0.026	1.178 ± 0.041	1.213 ± 0.033	1.273 ± 0.010	1.558 ± 0.029**	
Relative	43.949 ± 1.099	42.829 ± 0.597	46.503 ± 0.763*	51.263 ± 0.450**	64.188 ± 1.084**	
Lung						
Absolute	0.247 ± 0.011	0.241 ± 0.010	0.260 ± 0.022	0.245 ± 0.014	0.209 ± 0.011	
Relative	8.482 ± 0.499	8.793 ± 0.297	9.861 ± 0.579	9.873 ± 0.536	8.612 ± 0.429	
Thymus						
Absolute	0.049 ± 0.003	0.055 ± 0.003	0.053 ± 0.003	0.048 ± 0.002	0.054 ± 0.002	
Relative	1.682 ± 0.088	2.016 ± 0.111*	2.019 ± 0.077*	1.944 ± 0.088*	2.243 ± 0.113**	

* Significantly different (P≤0.05) from the vehicle control group by Williams' or Dunnett's test
** P≤0.01
a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX E
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF ESTRAGOLE

Estragole was obtained from Penta International Corporation (Livingston, NJ) in one lot (57533). The analytical chemistry laboratory, Battelle Columbus Operations (Chemistry Support Services, Columbus, OH), performed vacuum distillation on lot 57533, and the distilled product was assigned lot number 021401 and was used during the 3-month studies. Identity and purity analyses were conducted by the analytical chemistry laboratory and the study laboratory, Battelle Columbus Operations (Columbus, OH). Karl Fischer titration and elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). Reports on analyses performed in support of the estragole studies are on file at the National Institute of Environmental Health Sciences.

Lot 021401, a clear, colorless liquid, was identified as estragole by the analytical chemistry laboratory using infrared and proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy; the study laboratory confirmed the identity of the test article by infrared spectroscopy. All spectra were consistent with the structure of estragole, spectra from a previous analysis and from a frozen reference standard of the same lot, a literature reference (*Aldrich*, 1997), and/or computer calculated spectra (NMR). Representative infrared and proton and carbon-13 NMR spectra are presented in Figures E1, E2, and E3.

The purity of lot 021401 was determined by the analytical chemistry laboratory using gas chromatography (GC) by system A and high performance liquid chromatography (HPLC) using a Hewlett Packard (Palo Alto, CA) instrument with a Prodigy® 5 ODS-3 column (150 mm × 4.6 mm; Phenomenex, Torrance, CA) and a mobile phase of A: 50:50:1 acetonitrile:Milli-Q® water:glacial acetic acid and B: 90:10:1 acetonitrile:Milli-Q® water:glacial acetic acid, held at 100% A for 7 minutes, then linear to 100% B in 1 minute, held at 100% B for 10 minutes, then linear to 100% A in 0.1 minute, held at 100% A for 9.9 minutes; the flow rate was 1 mL/minute with ultraviolet detection at 270 nm. The study laboratory determined purity using GC by system B. The analytical chemistry laboratory and the study laboratory determined the peroxide concentration of lot 021401 using potentiometric titration.

A) Hewlett-Packard gas chromatograph, an RTX-5 (30 m × 0.53 mm, 1.5-µm film thickness) column (Restek, Bellefonte, PA), flame ionization detection, helium as a carrier gas at a flow rate of 5 mL/minute, and an oven temperature program of 50° C for 3 minutes, then 10° C/minute to 300° C, held for 2 minutes

B) Hewlett-Packard gas chromatograph, a Stabilwax® Crossbond® (30 m × 0.25 mm, 0.25-µm film thickness) column (Restek), flame ionization detection, helium as a carrier gas at a flow rate of approximately 3 mL/minute, and an oven temperature program of 80° C for 2 minutes, then 15° C/minute to 240° C, held for 9 minutes

Karl Fischer titration indicated 194 ppm water. Elemental analyses for carbon, hydrogen, and oxygen were in agreement with the theoretical values for estragole. Gas chromatography by system A indicated one major peak and no impurities greater than 0.1% of the total peak area; purity was determined to be greater than 99%. Gas chromatography by system B indicated a purity of 99.5% relative to a frozen reference sample of the same lot. HPLC indicated one major peak and no impurities greater than 0.1% of the total peak area. The peroxide concentration was below the acceptable limit of 3 mEq/kg. The overall purity of lot 021401 was determined to be greater than 99%.

To ensure stability, the bulk chemical was stored in sealed amber glass bottles under an argon headspace, protected from light, at approximately –20° C. Reanalysis of the bulk chemical was performed twice during the studies by the study laboratory using GC by system B and potentiometric titration to determine peroxide concentrations. No degradation of the bulk chemical was detected, and peroxide concentrations were within acceptable limits.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared by mixing estragole with corn oil (Table E1). The dose formulations were stored at 5° C in amber glass bottles under an argon headspace, sealed with Teflon®-lined lids for up to 42 days.

The analytical chemistry laboratory performed homogeneity studies (0.2 and 120 mg/mL dose formulations), a gavageability study (120 mg/mL dose formulation), and stability studies (0.2 mg/mL dose formulation) using GC by a system similar to system B with a carrier gas flow rate of approximately 2.5 mL/minute. Homogeneity and gavageability were confirmed, and stability was confirmed for at least 42 days for dose formulations stored in amber glass bottles protected from light at approximately –20°, 5°, or 25° C and for at least 3 hours under simulated animal room conditions.

Periodic analyses of the dose formulations of estragole were conducted by the study laboratory using GC by system B. During the 3-month studies, the dose formulations were analyzed three times; animal room samples of these dose formulations were also analyzed (Table E2). All dose formulations prepared for rats (15) and mice (15) were within 10% of the target concentrations; all animal room samples analyzed for rats (15) and mice (15) were within 10% of the target concentrations. Corn oil was obtained in two lots (QQ0053 and QN0035) from Spectrum Chemicals and Laboratory Products (Gardena, CA) and was used as the vehicle during the 3-month studies. The study laboratory determined peroxide concentrations three times during the studies using potentiometric titration; all peroxide concentrations were below the acceptable limit of 3 mEq/kg.

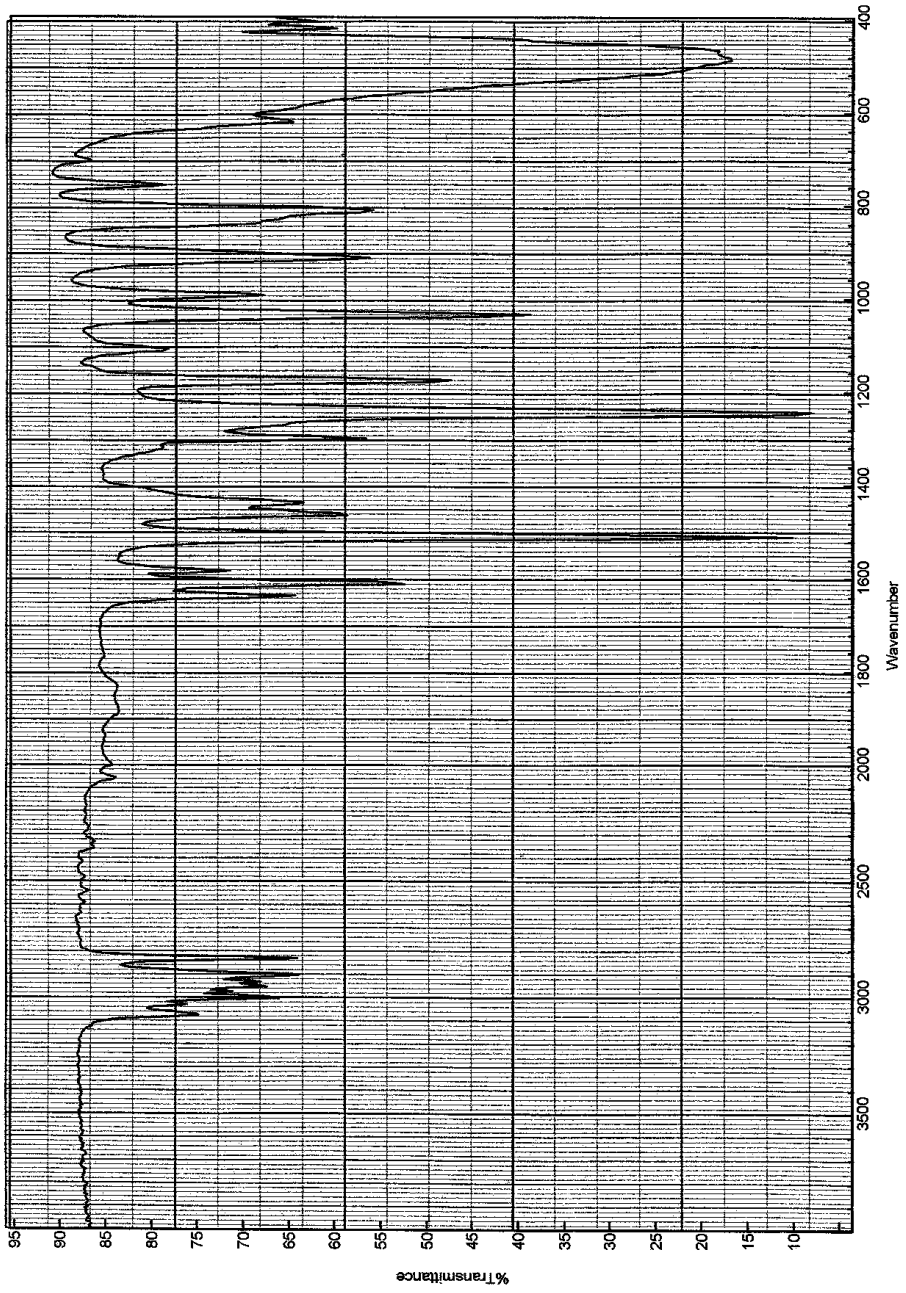

FIGURE E1
Infrared Absorption Spectrum of Estragole

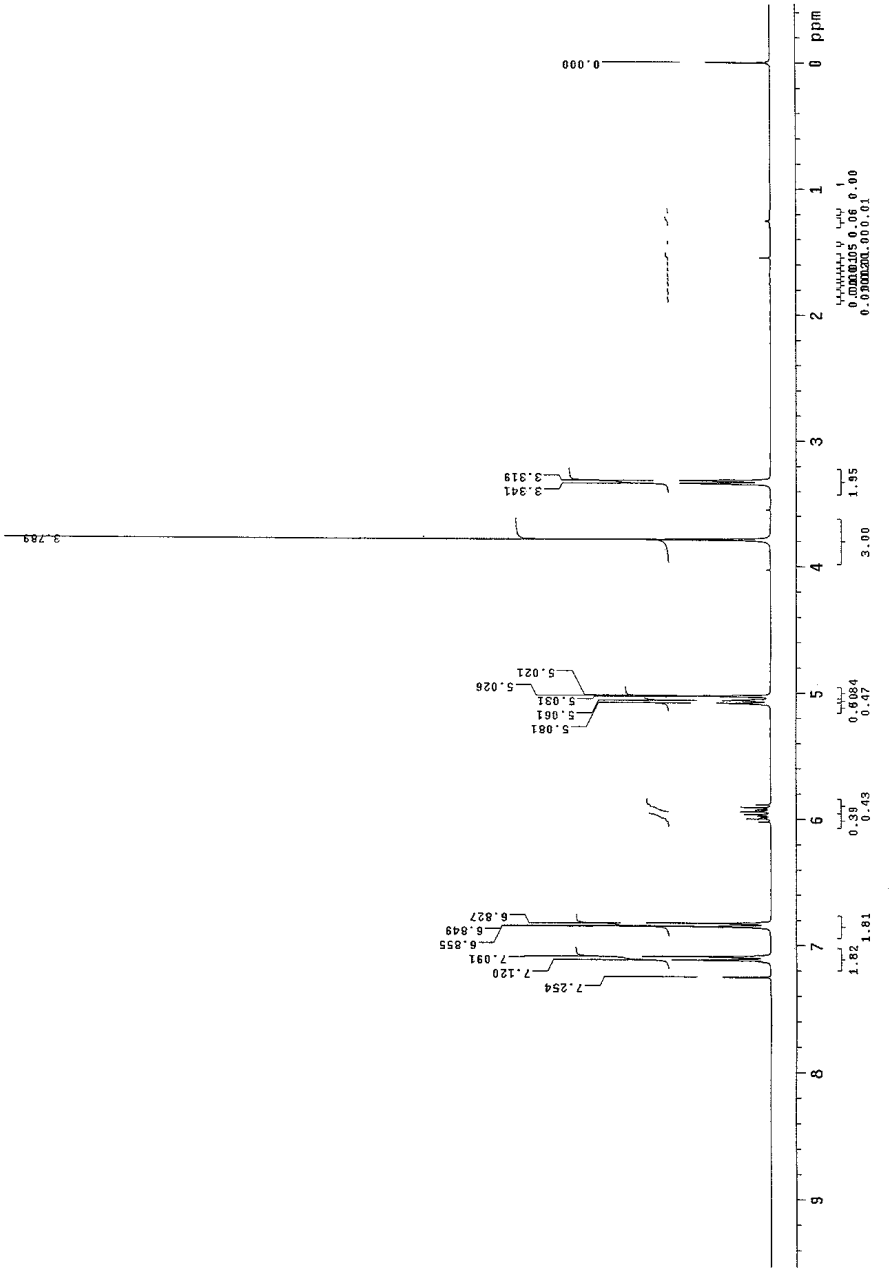

FIGURE E2
Proton Nuclear Magnetic Resonance Spectrum of Estragole

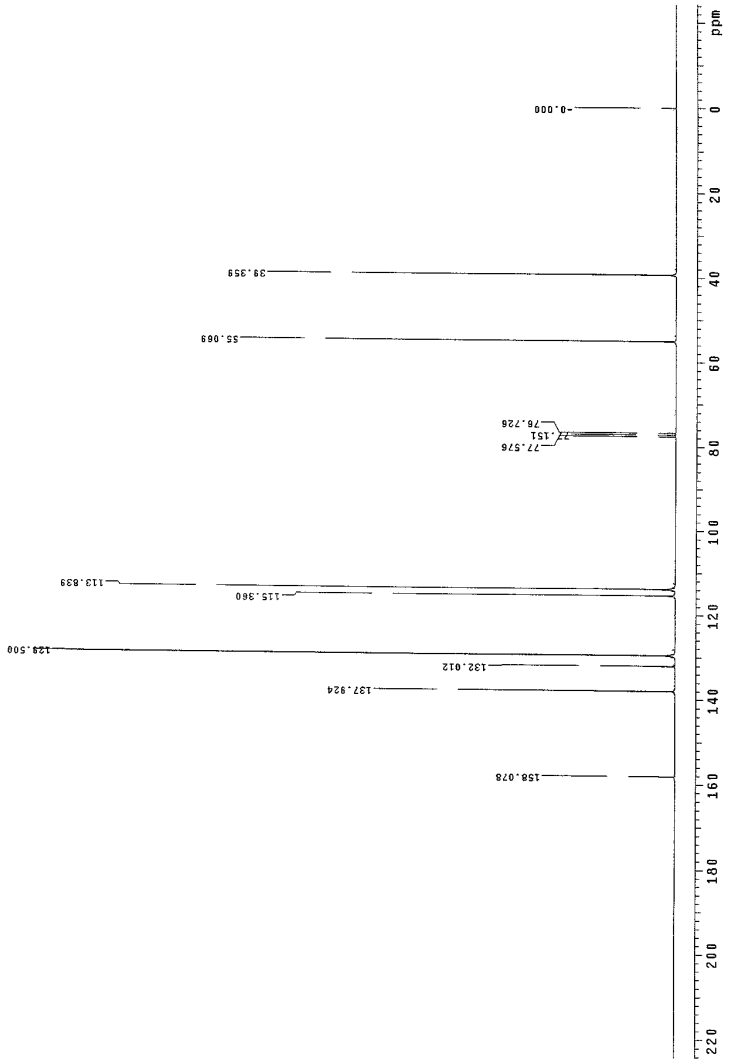

FIGURE E3
Carbon-13 Nuclear Magnetic Resonance Spectrum of Estragole

TABLE E1
Preparation and Storage of Dose Formulations in the 3-Month Gavage Studies of Estragole

Preparation
The appropriate amounts of estragole and corn oil were placed in a glass mixing container, capped, and thoroughly mixed with a paint shaker for approximately 2 minutes. Dose formulations were prepared three times during the 3-month studies.

Chemical Lot Number
021401

Maximum Storage Time
42 days

Storage Conditions
Stored in amber glass bottles under a headspace of argon gas sealed with Teflon®-lined lids at approximately 5° C

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

TABLE E2
Results of Analyses of Dose Formulations Prepared for Rats and Mice in the 3-Month Gavage Studies of Estragole[a]

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Rats				
August 29, 2001	August 31-September 6, 2001	7.5	8.032	+7
		15	15.29	+2
		30	30.70	+2
		60	59.73	0
		120	117.5[b]	–2
	October 5-6, 2001[c]	7.5	7.723	+3
		15	15.03	0
		30	29.42	–2
		60	59.86	0
		120	122.8	+2
September 24 or 26, 2001	September 28-29, 2001	7.5	7.723	+3
		15	15.07	0
		30	30.15	+1
		60	61.06	+2
		120	123.6	+3
	October 31-November 1, 2001[c]	7.5	7.667	+2
		15	15.15	+1
		30	31.63	+5
		60	61.82	+3
		120	124.7	+4
November 12, 2001	November 13-14, 2001	7.5	7.860	+5
		15	15.11	+1
		30	31.17	+4
		60	61.63	+3
		120	125.1	+4
	December 18-19, 2001[c]	7.5	7.859	+5
		15	15.60	+4
		30	31.21	+4
		60	61.32	+2
		120	125.7	+5

TABLE E2
Results of Analyses of Dose Formulations Prepared for Rats and Mice in the 3-Month Gavage Studies of Estragole

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Mice				
August 29, 2001	August 31-September 1, 2001	7.5	8.032	+7
		15	15.29	+2
		30	30.70	+2
		60	59.73	0
September 4, 2001	September 6, 2001	3.75	4.041	+8
	October 5-6, 2001[c]	3.75	3.804	+1
		7.5	7.650	+2
		15	15.17	+1
		30	29.75	–1
		60	58.87	–2
September 24 or 26, 2001	September 28-29, 2001	3.75	3.801	+1
		7.5	7.723	+3
		15	15.07	0
		30	30.15	+1
		60	61.06	+2
	October 31-November 1, 2001[c]	3.75	3.892	+4
		7.5	7.655	+2
		15	15.22	+1
		30	30.11	0
		60	61.72	+3
November 12 or 16, 2001	November 13-14 or 20-21, 2001	3.75	3.876	+3
		7.5	7.860	+5
		15	15.11	+1
		30	31.17	+4
		60	61.63	+3
	December 18-19, 2001[c]	3.75	3.867	+3
		7.5	7.812	+4
		15	15.45	+3
		30	30.57	+2
		60	61.38	+2

[a] Results of duplicate analyses. For rats, dosing volume =5 mL/kg; 7.5 mg/mL=37.5 mg/kg, 15 mg/mL=75 mg/kg, 30 mg/mL=150 mg/kg, 60 mg/mL=300 mg/kg, 120 mg/mL=600 mg/kg. For mice, dosing volume=10 mL/kg; 3.75 mg/mL=37.5 mg/kg, 7.5 mg/mL=75 mg/kg, 15 mg/mL=150 mg/kg, 30 mg/mL=300 mg/kg, 60 mg/mL=600 mg/kg.

[b] Result of quadruplicate analyses

[c] Animal room samples

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