Table IV. Toxicities of Croneton and Its Oxidized Sulfur Analogues to Rats and Mice[a]

Compound	Approximate LD_{50}, mg/kg[b]		LD_{50}, mg/kg,[c]
	Rats	Mice	mice
Croneton	338	150	71
Croneton sulfoxide	150	67	59
Croneton sulfone	506	225	282

[a] Twenty-four h LD_{50} following single oral dose. [b] Determined by the approximation method of Deichmann and LeBlanc (1943). [c] Determined by log dosage/probit analyses of Litchfield and Wilcoxon (1949).

Thanks also are extended to C. Rieck, Department of Agronomy, University of Kentucky, for his helpful discussion and suggestions, and to Susan Lolmaugh of our laboratory for her very able technical assistance.

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Received for review September 15, 1975. Accepted November 17, 1975. This study was supported by a grant-in-aid from Chemagro, Agricultural Division of Mobay Chemical Corp. and, in part, from Environmental Protection Agency Grant No. R802005, and Regional Research Project S-73.

Metabolism of (+)-Limonene in Rats

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(+)-Limonene was administered intragastrically to rats and ten terpenoid metabolites were isolated from the urines. Of these metabolites, seven were identified by chromatographic and spectral analysis as *p*-mentha-2,8-dien-1-α-ol, *p*-mentha-2,8-dien-1-β-ol, *p*-mentha-1,8-dien-6-α-ol, *p*-mentha-1,8-dien-6-β-ol, *p*-mentha-2-ene-8,9-diol, *p*-mentha-1,8-dien-7-ol, and 4-isopropenyl-1-cyclohexene-1-carboxylic acid.

(+)-Limonene (*p*-mentha-1,8-diene) is a major component of the essential oils of many fruits and spices. (+)-Limonene and essential oils containing (+)-limonene are used extensively in the food and cosmetic industries. Until recently, however, little was known about the metabolism of this terpene in mammals.

In 1966, Wade et al. reported the isolation of uroterpenol (*p*-mentha-1-ene-8,9-diol) from human urine. Subsequent studies by Smith et al. (1969) showed that ingestion of limonene resulted in increased levels of uroterpenol in human urine.

Recently, two studies of the metabolism of (+)-limonene in rabbits and rats have been reported. Kodama et al. (1974) fed radioactive limonene to rabbits and found that 72% of the radioactivity appeared in the urine and 7% in the feces. Eight metabolites were indicated by thin-layer chromatography of the radioactive urine extract. Of the eight, Kodama isolated and identified six metabolites: *p*-mentha-1,8-dien-10-ol, uroterpenol, perillic acid (4-isopropenyl-1-cyclohexene-1-carboxylic acid), a dihydroxyperillic acid, *p*-mentha-1,8-dien-10-ol glucuronide, and uroterpenol glucuronide.

In the rat study Igimi et al. (1974) fed radioactive limonene and followed its absorption and excretion by analysis of urine, feces, bile, and expired air. The excretion of the radioactivity occurred predominantly in the urine (58% of the dose) with feces and bile making up 5.1 and 25%, respectively. Igimi et al. (1974) noted that thin-layer chromatography of the radioactive rat urine extracts indicated the presence of at least ten metabolites. By using comparative thin-layer chromatography these workers identified four metabolites: uroterpenol, perillic acid, 8,9-dihydroxyperillic acid, and uroterpenol glucuronide.

The purpose of this investigation was to isolate and identify the nonconjugated urinary metabolites of limonene in rats.

MATERIALS AND METHODS

(+)-Limonene obtained from J. T. Baker Chemical Co. was purified to greater than 99% by a combination of fractional distillation at reduced pressure (27 mmHg; 75°C) and by preparative gas–liquid chromatography (GLC). Uroterpenol was synthesized from (+)-limonene using the method described by Dean et al. (1967) and purified by silica gel column chromatography to greater than 99%, as judged by GLC. Perillyl alcohol was prepared from β-pinene using the method of Sato (1965) and purified by preparative GLC. Silica gel 60 (particle size less than 0.63 mm) was purchased from E.M. Reagents and used for column chromatography. Reagent grade solvents were used.

Animals and Dosing. Adult male Long-Evans rats (500–600 g) were used. Ten rats were each fed 0.25 ml of limonene per day for 10 days by pharynogogastric intubation. This dose of limonene corresponds to a dose of 40 mg/kg and the total amount of limonene fed to all the rats equaled 21 g. The animals were housed in individual stainless steel metabolism cages and they were allowed food (Purina Rat Chow) and water ad libitum.

Gas–Liquid Chromatography (GLC). A Varian Aerograph 200 gas chromatograph was used in conjunction with a Honeywell recorder and a Hewlett Packard 50B automatic attenuator. The chromatograph had a thermal conductivity detector. The purification of limonene was carried out on a $^3/_8$ in. × 20 ft preparative column containing 20% FFAP (Carbowax 20M plus 10% nitroterophthalic acid) on 40/60 mesh Chromosorb A (Varian Aerograph). The conditions used were as follows: injection

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temperature, 220°C; column temperature, 195°C; detector temperature, 220°C; helium flow rate, 150 ml/min helium at 60 psi pressure.

For the detection of nonpolar neutral metabolites the same chromatograph was used with the following column: 0.25 in. × 5 ft analytical column of 10% Apiezon L, on 60/80 mesh Chromosorb W (Varian Aerograph) treated with dimethylchlorosilane (DMCS). The conditions for the nonpolar neutral metabolites were as follows (hereafter these conditions will be referred to as the *neutral conditions*): injection temperature, 200°C; column temperature, 120°C; detector temperature, 210°C; helium flow rate, 100 ml/min at 60 psi.

For the acidic metabolites a 0.25 in. × 5 ft analytical column of 2.5% FFAP on 80/100 mesh Chromosorb G (Varian Aerograph, DMCS treated) was used. The temperatures of the injection port, column, and detector were maintained at 215, 175, and 220°C, respectively, with a helium flow rate of 100 ml/min at 60 psi. These conditions will hereafter be referred to as the *acidic conditions*.

Thin-Layer Chromatography (TLC). TLC plates (5 × 10 and 5 × 20 cm) manufactured by E. Merck were obtained from Brinkmann Instruments Inc. The plates were glass, precoated with silica gel 60, F-245, and were of 0.25 mm thickness. Two solvent systems of varying polarity were employed for development. System A consisted of ethyl acetate–cyclohexane (20:80, v/v). System B contained ethyl acetate–ethanol–cyclohexane (20:30:50, v/v/v), plus five drops of acetic acid per 100 ml of solvent. The chromatograms were observed under short-wavelength ultraviolet light and the compounds were visualized by spraying the chromatograms with 1% vanillin in 70% sulfuric acid, followed by heating at 115°C for 5–10 min. Normal products of rat metabolism (i.e., untreated rat urine) gave colors varying from yellow to light brown to a very dark brown. Monoterpenes arising from the metabolism of administered limonene gave colors ranging from light blue to violet. Limonene exhibits a faint red brown color under these conditions.

Spectra. Infrared spectra were recorded on a Perkin-Elmer Model 137 spectrophotometer from chloroform solutions. A Varian Associates T-60 was used to record NMR spectra, and low-resolution mass spectra were recorded with an AEI MS-12 at 70 eV using the direct inlet.

Miscellaneous. Melting points were determined in open capillary tubes with a Buchi melting point apparatus and they were not corrected.

Urine Collection. Urine was collected at 0–5°C to inhibit both bacterial growth and endogenous β-glucuronidase activity. This was accomplished by tapping the urine collecting funnel on each metabolism cage with rubber tubing. The unattached end of the rubber tubing was then placed over a funnel which drained into a 500-ml Erlenmeyer flask, packed in ice. Urine was collected, and the cages and tubing cleaned, every 48 h. The volume of urine collected during a 48-h period for ten rats was 300 ± 50 ml. For a single dosing schedule (0.25 ml of limonene per rat per day for 10 days), urine collection was started immediately after the administration of the first dose and continued for 12 days (48 h after the last dose).

Extraction of Urinary Metabolites. The following extraction procedure was used for both untreated and limonene treated rat urines. The urine (300 ml) was acidified with concentrated HCl to a pH of 2.0 and extracted with three 100-ml portions of chloroform to remove less polar neutral and acidic metabolites. The chloroform extracts were then washed with two 150-ml portions of a saturated solution of sodium bicarbonate to remove acidic metabolites. The chloroform phases containing the remaining neutral material (neutral extract) were then concentrated under reduced pressure in a rotary evaporator. The bicarbonate phases were acidified with HCl to a pH of 2.0 and extracted with three 100-ml portions of chloroform to remove the acidic metabolites. The acidified bicarbonate was discarded and the chloroform extracts (acidic extract) were concentrated.

GLC of Whole Extracts. Urine (300 ml) from both limonene treated rats and untreated rats was extracted according to the previously mentioned procedure within 1 day after collection of the urine. The neutral and acidic extracts were concentrated to 1.0 ml, and 25 μl of each extract was used for GLC analysis. For the neutral extracts the neutral conditions were used and for the acidic extracts the acidic conditions were employed.

RESULTS

Neutral Extract; Detection and Isolation of Metabolites. Portions of the neutral extract from both limonene treated and untreated rat urines were subjected to thin-layer chromatography (systems A and B). TLC indicated the presence of at least four substances in the urine from the limonene treated rats, which were absent from the urines of untreated rats.

The neutral extract from the limonene treated rats (0.60 g from 900 ml of urine) was dissolved in 2.0 ml of cyclohexane–ethyl acetate (85:15, v/v) and applied to a column of silica gel (1.0 cm × 35.0 cm) which had been packed in the same solvent system. The column was eluted successively with 100 ml of cyclohexane–ethyl acetate (85:15, v/v), 100 ml of cyclohexane–ethyl acetate (80:20, v/v), and 100 ml of cyclohexane–ethyl acetate (60:40, v/v).

The first 100-ml eluate was concentrated and two metabolites, LM-1 and LM-2, were isolated by GLC using the neutral conditions. Approximately 10–12 mg of LM-1 and 20 mg of LM-2 were obtained. They are both colorless oils with retention times of 5.9 and 10.2 min, respectively.

The second 100-ml eluate was concentrated and one metabolite, LM-3, was isolated by GLC using the neutral conditions. A total of 18 mg of LM-3 was obtained. It has a GLC retention time of 6.3 min and is a colorless oil.

The third 100-ml eluate was concentrated and two metabolites, LM-8 and LM-9, were isolated by GLC using the polar conditions. They had retention times of 3.5 and 6.7 min, respectively, on GLC. Approximately 6 mg each of LM-8 and LM-9 was obtained as oils which crystallized upon standing overnight.

The column was eluted a fourth time with 100 ml of cyclohexane–ethyl acetate (60:40, v/v) and upon evaporation of the solvent, 25–30 mg of a sixth metabolite, LM-6, was obtained. LM-6 is a colorless oil and was found to have a GLC retention time of 13.5 min (polar conditions).

Acidic Extract; Detection and Isolation of Metabolites. TLC analysis (system B) of the acidic extract from both limonene treated rats and untreated rats revealed at least two new compounds in the extract from the limonene treated rats.

Column chromatography on silica gel was used to isolate the two acidic metabolites. Approximately 1.0 g of the concentrated acidic extract (from 900 ml of urine) was placed on a column (70 cm × 1.5 cm) of silica gel packed in cyclohexane–ethyl acetate–ethanol (70:15:15, v/v/v) and the column was eluted with 500 ml of the same solvent system. The first compound, LM-7, was recrystallized from a mixture of cyclohexane and petroleum ether and further purified by sublimation (85°C, 0.5 mmHg). LM-7

Table I. Chemical Shifts in the Nuclear Magnetic Resonance Spectra of Limonene and Metabolites

Position	Proton shift[a] (+)-Limonene	LM-1	LM-2,4	LM-3	LM-5	LM-6	LM-7
C-2	5.4 m	5.6 s	5.4 m	5.6 m	5.6 m	5.3 m	7.1 m
C-3		5.6 s		5.6 m			
C-5			4.0 m				
C-8	4.7 s	4.7 m	4.8 m	4.6 m	4.7 s	3.2 m	4.7 s
C-9	1.6–1.8 s	1.7 m	1.8 m	1.7 m	1.8 s	1.1 d	1.8 s
C-10	1.6–1.8 s	1.2 s	1.8 m	1.2 s	3.9 s	1.7 s	

[a] Values for chemical shifts are given as parts per million (δ) downfield from the tetramethylsilane internal standard: d = doublet, m = multiplet, and s = singlet. Solvent is CCl_4, except for M-7 for which it was $CDCl_3$.

is a white crystalline compound with a melting point of 128–129°C. The second compound, LM-10, was recrystallized from ethyl acetate–carbon tetrachloride. LM-10 is a white crystalline substance with a melting point of 140–141°C.

Identification of Metabolites. Chromatographic purification of extracts of urine from rats intragastrically given (+)-limonene has resulted in the isolation of ten metabolites, seven of which have been identified by their spectral and chromatographic characteristics. Distinctive features of the NMR spectra of these metabolites and limonene are indicated in Table I.

Metabolite LM-1 has NMR and mass spectra [m/e 152 (M^+), 137 ($M^+ - CH_3$), 134 ($M^+ - H_2O$), 119 ($M^+ - CH_3 - H_2O$)] which are consistent with the p-mentha-2,8-dien-1-ol structural assignment. This assignment was confirmed by comparison of the infrared spectrum of LM-1 with a published spectrum of characterized material (Mitzner et al., 1968). The infrared data also established the configuration of the C-1 hydroxyl group as α.

Metabolite LM-3 has NMR, infrared, and mass spectra [m/e 152 (M^+), 137 ($M^+ - CH_3$), 134 ($M^+ - H_2O$), 119 ($M^+ - CH_3 - H_2O$)] which are very similar to the corresponding spectra of LM-1. The identity of LM-3 is confirmed and the configuration of the C-1 hydroxyl group is established as β by comparison of the infrared spectrum of LM-3 with a published spectrum (Mitzner et al., 1968) of p-mentha-1,8-dien-1-β-ol.

Metabolites LM-2 and LM-4 were isolated as a mixture (as established by GLC) with NMR and mass spectra [m/e 152 (M^+), 134 ($M^+ - H_2O$)] consistent with the p-mentha-1,8-dien-6-ol (carveol) structural assignment. Comparison of the infrared and NMR spectra of the isolated material with the corresponding published spectra (Mitzner et al., 1968; Dhavalikar and Bhattacharyya, 1966) of p-mentha-1,8-dien-6-α- and -β-ol confirms the proposed identity of these metabolites.

Metabolite LM-6 was identified as p-mentha-2-ene-8,9-diol (uroterpenol) by comparison of its NMR and infrared spectra and GLC and TLC behavior with corresponding data of an authentic sample obtained by synthesis (Dean et al., 1967). The spectra and chromatographic properties of LM-6 were indistinguishable from those of the synthetic material. The NMR spectra of the synthetic and metabolic material indicate they are diastereoisomeric mixtures as suggested by Dean et al. (1967). However, this mixture was not resolvable by the GLC system used in the present study.

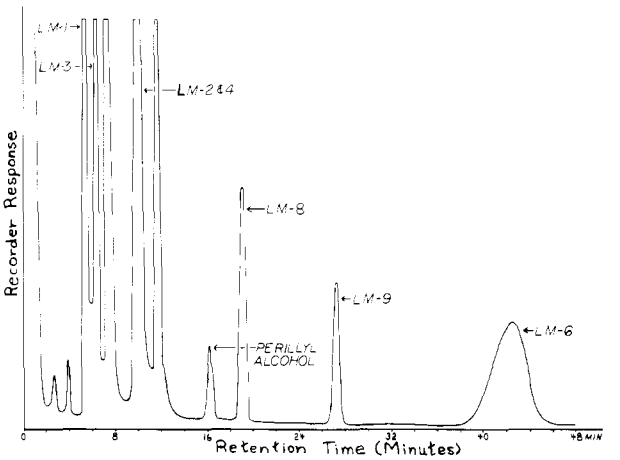


Figure 1. Gas-liquid chromatogram of neutral extract using neutral conditions.

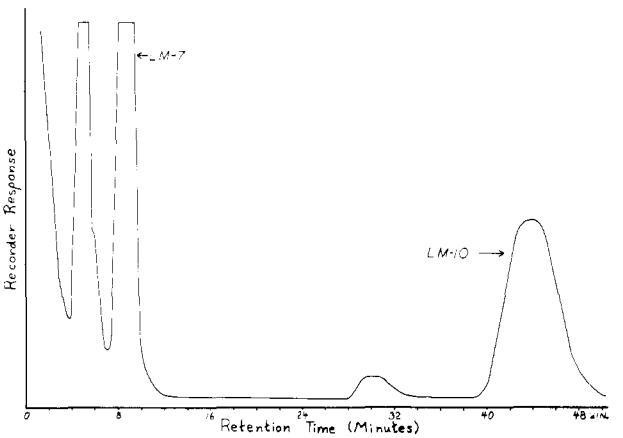


Figure 2. Gas-liquid chromatogram of polar metabolites using acidic conditions.

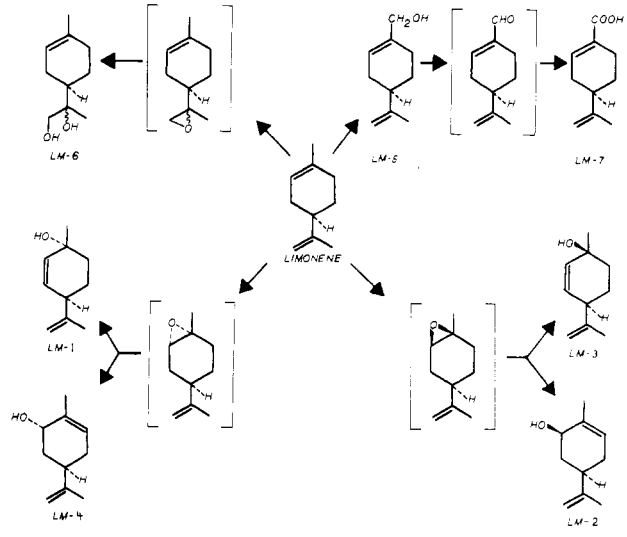
Metabolite LM-5 was present in very small quantities in the urines of limonene-treated animals and was identified by coinjection on GLC of the urine extract with a synthetic sample of p-mentha-1,8-dien-7-ol (perillyl alcohol) (Sato, 1965). This analysis produced a single GLC peak with retention time (1.34 min) identical with that of the synthetic material.

Metabolite LM-7 has NMR, infrared, and mass spectra [m/e 166 (M^+), 148 ($M^+ - H_2O$), 121 ($M^+ - CO_2H$)] which are consistent with the 4-isopropenyl-1-cyclohexene-1-carboxylic acid (perillic acid) structural assignment. The NMR and infrared spectra and melting point of LM-7 are identical with corresponding published data of perillic acid (Dhavalikar and Bhattacharyya, 1966).

In addition to the metabolites described above, three unidentified compounds (LM-8, LM-9, and LM-10) were isolated. LM-8 and LM-9 are polyalcohols and LM-10 is a hydroxylated carboxylic acid as indicated by NMR and solubility characteristics. Further characterization of these compounds is in progress.

GLC of Whole Extracts. The results of the GLC of whole extracts are presented in Figures 1 and 2. Assuming that the major unconjugated metabolites of limonene have been accounted for and detector responses for the metabolites are similar, the relative excretion of each metabolite was calculated based on the GLC data. It was observed that the major metabolites are *cis*- and *trans*-p-mentha-2,8-dien-1-ol (LM-1, LM-3, 37%), perillic acid (LM-7, 24.6%), and the carveol isomers (LM-2, LM-4, 16.2%). Smaller amounts of uroterpenol (LM-6, 5.1%) and perillyl alcohol (LM-5, 0.4%) appear in the urine. The

Scheme I. Proposed Metabolic Pathway of Limonene in Rats



unidentified compounds (LM-8, LM-9, and LM-10) comprise 17.5% of the total metabolites. (+)-Limonene was not detected in this analysis.

DISCUSSION

The results of this study have demonstrated the presence of eight neutral metabolites and two acidic metabolites in the urine of limonene-fed rats. Of the ten compounds obtained in the present study only two have been mentioned in the previous metabolic investigations in mammals. A total of 14 different metabolites have now been shown to result from the metabolism of limonene in higher organisms.

The results of the work of Kodama et al. (1974) and of Igimi et al. (1974), as well as the present study, show at least three common metabolites for rats and rabbits; these are uroterpenol, uroterpenol glucuronide, and perillic acid. In addition, the finding of uroterpenol in these three studies corroborates the evidence of Wade et al. (1966) that dietary limonene gives rise to the uroterpenol that occurs in human urine. In addition to the presence of uroterpenol, uroterpenol glucuronide, and perillic acid, Igimi et al. (1974) also report the occurrence of 8,9-dihydroxy perillic acid in rat urine. However, we have no indication of the presence of the latter compound in rat urine.

The metabolites isolated in this study represent formal allylic and direct oxidations of the double bonds of limonene. Perillic acid (LM-7) and perillyl alcohol (LM-5) appear to arise by consecutive, allylic oxidations of C-7 of limonene. Compounds LM-1,3, LM-2,4, and LM-7 are three sets of diastereoisomers, which could be formed from limonene via the appropriate epoxides. These pathways are represented in Scheme I. Proposed intermediates are indicated in brackets and stereochemical assignments for the metabolites are relative. The possible role of epoxides A and B in limonene metabolism is being investigated.

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Received for review August 11, 1975. Accepted November 10, 1975.

A New Liquid Chromatography Approach to Plant Phenolics. Application to the Determination of Chlorogenic Acid in Sunflower Meal

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A new technique developed for trace analysis of phenolic compounds combines the advantages of high performance liquid chromatography with thin-layer electrochemistry. Liquid chromatography with amperometric detection at a carbon paste electrode is shown to be ideally suited to the determination of chlorogenic acid in plant matter. Due to the low oxidation potential of chlorogenic acid, it is possible to quantitatively measure a little as 25 pg by chromatography on a polyamide stationary phase (50 cm × 2.1 mm) using 0.1 M citric acid as the mobile phase. A sample of commercial sunflower meal was found to contain 2.6 ± 0.1% (SD) of chlorogenic acid based on the dry weight of defatted meal.

A new approach to the trace analysis of phenolic compounds and aromatic amines has been under development in our laboratory. This approach involves the combination of high performance liquid chromatography with electrochemical (amperometric) detection (LCEC). The emphasis up to this point has been on the analysis of drugs and metabolites in body fluids (Kissinger et al., 1974, 1976; Riggin et al., 1975). This new technique has also proven to be invaluable for the assay of pharmaceutical dosage forms (Riggin et al., 1974), ascorbic acid in pharmaceuticals and food products (Pachla and Kissinger, 1976), and isoquinoline alkaloids in plant matter (Riggin et al., 1976). The fact that many of the phenolic compounds in urine are of dietary origin led us to explore the direct applicability of LCEC to phenolic acids in plant material. In the present paper we briefly review the

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