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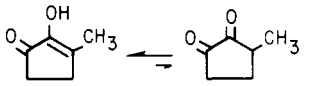
Quantitative Analysis of Alkyl-2-hydroxy-2-cyclopenten-1-ones in Tobacco Smoke

Stephen S. Hecht,* Steven Carmella, and Dietrich Hoffmann

2-Hydroxy-3-methyl-2-cyclopenten-1-one-*3-methyl*-^{14}C (1-*3-methyl*-^{14}C) was synthesized and employed as an internal standard for the quantitative analysis of 1 and related alkyl-2-hydroxy-2-cyclopenten-1-ones in cigarette smoke. The mainstream smoke of a typical U.S. blended 85-mm nonfilter cigarette contained 52 ± 3 μg of 1 as well as 3,5-dimethyl-2-hydroxy-2-cyclopenten-1-one (9.9 μg), 3,4-dimethyl-2-hydroxy-2-cyclopenten-1-one (2.1 μg), and 3-ethyl-2-hydroxy-2-cyclopenten-1-one (24 μg). The level of 1 in cigarette smoke was selectively reduced by cellulose acetate filter tips. The smoke of cigarettes made from Bright tobacco contained more 1 than observed in the smoke of Burley or Oriental cigarettes. Levels of 1 in smoke were reduced by extraction of tobacco with hexane–ethanol or by use of tobacco from the bottom stalk position. A correlation between concentrations of 1 and catechol in cigarette smoke was observed, indicating common leaf precursors for these two components.

Alkyl-2-hydroxy-2-cyclopenten-1-ones have been detected in tobacco smoke by several groups (Elmenhorst, 1972; Morêe-Testa and De Salles de Hys, 1975; Hecht et al., 1975; Schumacher et al., 1977; Sakuma and Sugawara, 1979). 2-Hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene, 1) is the most abundant compound in this group. It



1

exists predominantly as the enolic tautomer and is therefore detected in the weakly acidic or phenolic fractions of tobacco smoke (Son Bredenberg, 1959). We initially became interested in 1 because of its presence in subfractions of the weakly acidic fraction of cigarette smoke condensate which showed cocarcinogenic and tumor-promoting activity on mouse skin (Hecht et al., 1981). However, subsequent assays of 1 demonstrated that it was not active as a cocarcinogen or tumor promoter. The flavor properties of tobacco smoke, as well as numerous foods, are also influenced by 1, which has been described as having a sweet aroma and a sweet or maple taste (Elmenhorst, 1972; Leffingwell, 1972). Thus, the levels of 1 and related compounds in various products are important in determining their organoleptic properties. In the present study, we have developed a method for the quantitative analysis of 1 employing ^{14}C-labeled 1 as an internal standard.

EXPERIMENTAL SECTION

Reagents. All solvents were spectroquality. Ethyl 2-oxocyclopentanecarboxylate was obtained from Aldrich Chemical Co., Milwaukee, WI. $^{14}CH_3I$, 31.0 mCi/mmol, was procured from New England Nuclear, Boston, MA. TLC was performed with Merck precoated silica gel 60 F-254 plates.

Apparatus. Cigarettes were smoked on a Heinr. Borgwaldt Automatic Smoking Machine RM-20/68. GLC was performed with a Hewlett-Packard Model 5830A gas chromatograph equipped with a Model 18835B capillary inlet system, a flame ionization detector, and columns A [30 m × 0.25 mm Carbowax 20M (Supelco, Inc, Bellefonte, PA)] and B [6 ft × 2 mm glass column packed with 10% XE-60 on Chromosorb WHP]. GLC–MS was carried out with a Hewlett-Packard Model 5982A instrument. High-performance LC was done with a Waters Associates Model ALC/GPC-204 high-speed liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 660 solvent programmer, a Model U6K septumless injector, a Model 440 UV-visible detector, and a 3.9 mm × 30 cm μBondapak C_{18} column (Waters Associates, Milford, MA). Thin-layer radiochromatography was performed with a Packard Model 7201 radiochromatogram scanner. Scintillation counting was done with a Nuclear Chicago Isocap 300 system. UV spectra were run on a Cary Model 118 spectrometer.

Cigarettes. Cigarettes A–D were obtained on the open market in 1979. The Burley cigarettes of differing stalk positions were obtained through the courtesy of Dr. T. C. Tso, Tobacco Laboratory, U.S. Department of Agriculture, Beltsville, MD. The hexane–ethanol azeotrope extracted cigarettes (Gori, 1980), the corresponding unextracted cigarettes (standard experimental blend IV), and the modified cellulose cigarettes were made available by Enviro-Control, Rockville, MD.

2-Hydroxy-3-methyl-2-cyclopenten-1-one-*3-methyl*-^{14}C. A mixture of potassium ethyl 2-oxocyclopentanecarboxylate (3) (Mayer et al., 1958) (770 mg, 3.97 mmol) and $^{14}CH_3I$ (55.2 mg, 0.4 mmol, 5.0 mCi/mmol) in 1.5 mL of dry Me_2SO was stirred for 40 h at room tem-

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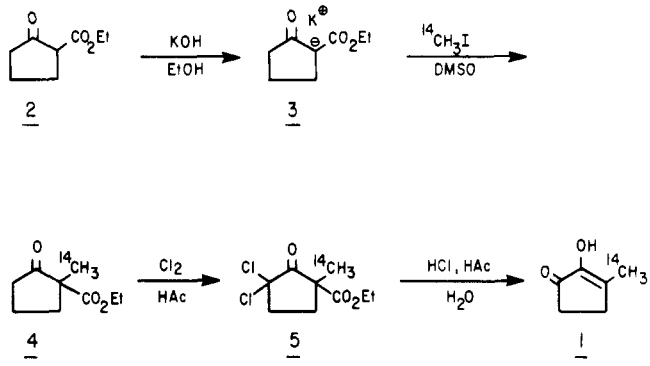
perature. The reaction was quenched by addition of 15 mL of H_2O and the resulting mixture was extracted with 7 × 15 mL of hexane. The combined organic extracts were washed with pH 10 aqueous base and dried (Na_2SO_4), and the hexane was removed by careful distillation to give 4, 43 mg, 65%, identical with unlabeled 4 prepared in the same way. To this was added a solution of Cl_2 (43 mg, 0.6 mmol) in 0.4 mL of glacial acetic acid. The volume was brought to 2 mL with glacial acetic acid, and the resulting solution was stirred for 16 h at room temperature. The acetic acid was removed under reduced pressure, leaving a residue of 5 which was used directly in the next step. A mixture of 18 mL of 1 N HCl and 1 mL of glacial acetic acid was added, and the solution was heated under reflux for 16 h. The resulting mixture was adjusted to pH 5 with 10 N NaOH and extracted with 5 × 20 mL of Et_2O. The combined Et_2O layers were washed with 50 mL of saturated aqueous $NaHCO_3$, dried (Na_2SO_4), and carefully concentrated to give crystalline 1-*3-methyl*-^{14}C (12 mg, 0.11 mmol, 27% based on $^{14}CH_3I$). The purity of 1 was greater than 95% according to analysis by thin-layer radiochromatography (silica, 50:50 benzene–Et_2O), high-performance LC using a gradient of 100% solvent A for 15 min and then linear to 30% solvent B in 15 min (solvent A was pH 3.9 acetate buffer and solvent B was 80% CH_3OH in H_2O) at a flow rate of 2 mL/min, and GLC on column B using a temperature program of 60 °C for 10 min and then 4 °C/min to 220 °C.

Smoke Analysis. Cigarettes and cigars were stored for at least 24 h in a humidity chamber at a relative humidity of 60 ± 3% and at 22 ± 2 °C. Smoking was done in a laboratory maintained at a relative humidity of 60 ± 5% at 22 ± 2 °C. The cigarette smoking conditions were one puff/min, puff duration of 2 s, puff volume of 35 mL, and butt length of 23 mm. For products with filter tips, butt length was determined by the filter plus overwrap plus 3 mm or was set at 23 mm, whichever was longer (Bates et al., 1968).

The mainstream smoke of 20 cigarettes was led through a 250-mL gas wash bottle cooled to −60 °C in a dry ice–ethylene glycol monomethyl ether bath. A filter holder with a 44-mm Cambridge CM-113 filter was placed between the gas wash bottle and the vacuum pump. After smoking was complete, the filter was washed with aqueous NaOH (0.1 N) and ether, and the washings were combined with the condensate.

The internal standard, 1-*3-methyl*-^{14}C (1 × 10^5 dpm, 1 μg), was added. The Et_2O and aqueous layers (60 mL each) were separated and cross-washed with 2 × 20 mL portions of Et_2O or 0.1 N NaOH. The combined aqueous layers were adjusted to pH 5.5–6.0 and extracted 4 times with equal volumes of Et_2O. The Et_2O layers were combined, dried (Na_2SO_4), and concentrated under reduced pressure at 20 °C to a final volume of 1–2 mL. The resulting Et_2O solution was applied to a single 20 × 20 cm preparative TLC plate (0.5- or 2.0-mm thickness) and developed with 70:30 benzene–Et_2O. The region of R_f 0.15–0.30, corresponding to 1 (R_f = 0.21) and catechol (R_f = 0.26) was scraped from the plate and sonically dispersed with Et_2O. The resulting mixture was filtered and concentrated to near dryness and the volume was adjusted to 1.0 mL. The radioactivity of a 50-μL aliquot was determined and a 5-μL aliquot was analyzed by capillary GLC using column A with a temperature program of 100 °C for 10 min and then 2 °C/min to 220 °C. Under these conditions the relative retention times of the alkyl-2-hydroxy-2-cyclopenten-1-ones were as follows: 3,5-dimethyl, 0.94; 3-methyl (1), 1.00; 3,4-dimethyl, 1.02; 3-ethyl, 1.13. The absolute retention time of 1 was 25.2 min.

Scheme I. Synthesis of 1-*3-methyl*-^{14}C



Tobacco Analysis. The tobacco of cigarette A (20 g) was stirred at 20 °C with 250 mL of 0.1 N NaOH for 16 h. The internal standard was added, the mixture was filtered, and the filtrate was analyzed according to the method described for smoke.

Selective Filtration. The smoke of cigarette C (from 50 mm of the tobacco column) was analyzed, and total particulate matter (TPM), nicotine, and H_2O were determined (Bates et al., 1968). The filter tips were then removed and an equivalent length of tobacco column (50 mm) was smoked under the same conditions, the smoke was analyzed, and TPM, nicotine, and H_2O were determined.

Catechol Analysis. Cigarette smoke was analyzed for catechol as previously described (Brunnemann et al., 1976) and by a newly developed method which will be described separately.

RESULTS AND DISCUSSION

The synthesis of ^{14}C-labeled 1 is outlined in Scheme I. This synthesis is based on a literature procedure for unlabeled 1 (Leir, 1970). Ethyl 2-oxocyclopentanecarboxylate (2) was converted to the corresponding potassium salt 3 and alkylated with $^{14}CH_3I$ to give ethyl 1-methyl-2-oxocyclopentanecarboxylate-*methyl*-^{14}C (4). Reaction of this keto ester with chlorine afforded ethyl 3,3-dichloro-1-methyl-2-oxocyclopentanecarboxylate-*methyl*-^{14}C (5), which was hydrolyzed with acid to 1-*3-methyl*-^{14}C. The latter reaction occurred in four steps: hydrolysis of the *gem*-dichloro function, hydrolysis and decarboxylation of the β-keto ester, and finally tautomerization to 1. The overall yield of 1-*3-methyl*-^{14}C from $^{14}CH_3I$ was 27%. The purity of the labeled compound was established by TLC, GLC, and high-performance LC.

For the analysis of 1 in cigarette smoke, we wanted to take advantage of its acidity. However, we suspected that 1 may not have been stable in base. To check this, we assayed the stability of 1 in aqueous NaOH by UV spectroscopy. The UV absorption of 1 was λ_{max} 256 nm at pH 6 and λ_{max} 293 nm at pH 12. The intensity of the latter absorption remained constant over a period of 60 min at room temperature. When the pH of the solution was readjusted to 6, λ_{max} 256 nm was again observed, without any significant decrease in intensity. We concluded that 1 had sufficient stability in base to allow adjustment to pH 12 in the analytical scheme.

The analytical method is summarized in Figure 1, and a typical GLC trace of the alkyl-2-hydroxy-2-cyclopenten-1-one fraction is shown in Figure 2. Under our conditions, good separations of 1, 3,4- and 3,5-dimethyl-2-hydroxy-2-cyclopenten-1-one, and 3-ethyl-2-hydroxy-2-cyclopenten-1-one were obtained. Each compound was identified by comparison of its GLC retention time and

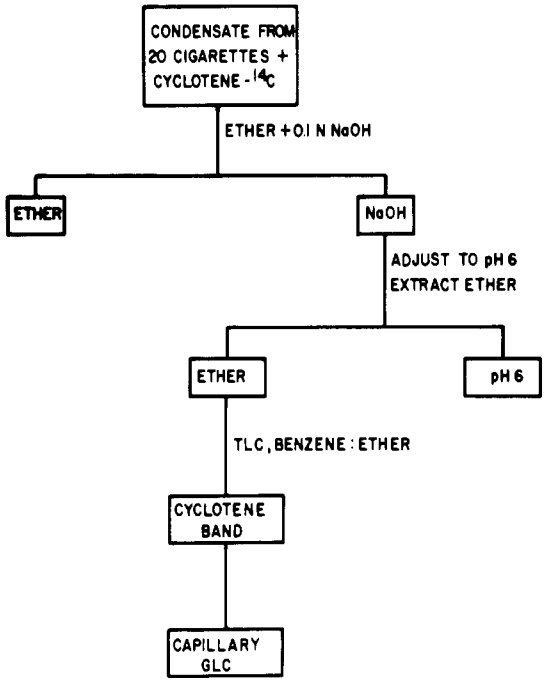


Figure 1. Analytical scheme for determination of alkyl-2-hydroxy-2-cyclopenten-1-ones in tobacco smoke (cyclotene = 1).

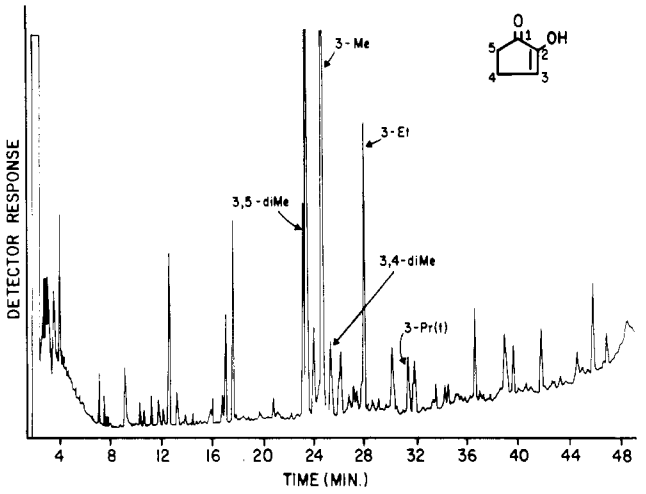


Figure 2. Gas chromatogram of the alkyl-2-hydroxy-2-cyclopenten-1-one fraction of tobacco smoke (t = tentative identification).

Table I. Alkyl-2-hydroxy-2-cyclopenten-1-ones in the Mainstream Smoke of Some Commercial Cigarettes

	TPM, mg	alkyl-2-hydroxy-2-cyclopenten-1-ones, μg/cigarette 3-Me	3,5-diMe	3,4-diMe	3-Et
(1) cigarette A, U.S. (NF)[a]	23.5	52[b]	9.9	2.1	24
(2) cigarette B, French (NF)[c]	24.4	33	5.7	1.4	22
(3) cigarette C, U.S. (F)[a,d]	19.3	6.1	0.16	0.12	3.5
(4) cigarette D, U.S. (F)[a]	8.0	1.0	ND[e]	ND	ND

[a] 85 mm. [b] Mean of three determinations; SD = 3 μg/cigarette. [c] 70 mm. [d] Cellulose acetate filter tip. [e] ND = not detected.

mass spectrum with that of a reference standard (Hecht et al., 1975).

Table II. Selective Filtration of Alkyl-2-hydroxy-2-cyclopenten-1-ones

	alkyl-2-hydroxy-2-cyclopenten-1-ones, μg/cigarette 3-Me	3-Et	TPM, mg
(1) cigarette C (without filter tip)[a]	54	36	25.5
(2) cigarette C (with filter tip)[a,b]	4.7	3.0	15.5

[a] 50 mm of 85-mm tobacco column smoked. [b] Cellulose acetate filter tip.

Table III. Alkyl-2-hydroxy-2-cyclopenten-1-ones in the Mainstream Smoke of Some Experimental Cigarettes[a]

	TPM, mg	alkyl-2-hydroxy-2-cyclopenten-1-ones, μg/cigarette 3-Me	3,5-diMe	3,4-diMe	3-Et
(1) reference IRI[b]	36	31	4.1	1.4	10
(2) Bright tobacco cigarette[c]	24	62	7.7	2.5	39
(3) Burley tobacco cigarette[c]	18	25	7.0	0.81	20
(4) Oriental tobacco cigarette[c]	25	50	8.2	NDe[d]	31
(5) std expt blend cigarette[b]	22	58	9.1	ND[e]	1.9
(6) std exptl blend cigarette, extracted[b,f]	10	10	2.4	0.76	1.7
(7) nontobacco cigarette[b]	5.0	3.7	ND	ND	ND

[a] Nonfilter cigarettes. [b] 85 mm. [c] 64 mm. [d] NDe = not determined due to interfering peak. [e] ND = not detected. [f] Extracted with a hexane-ethanol azeotrope.

Table I shows the quantitative values for 1 in the mainstream smoke of some commercial cigarettes. Recoveries ranged from 35 to 60%. The level of 1 in a typical U.S. blended 85-mm nonfilter cigarette (cigarette A) was similar to those observed in previous analytical studies on other blended cigarettes (Elmenhorst, 1972; Morée-Testa and De Salles de Hys, 1975). We did not detect 1 in the tobacco of cigarette A (detection limit, 0.05 μg/cigarette), in agreement with previous work (Elmenhorst, 1972; Morée-Testa and De Salles de Hys, 1975).

Since the level of 1 in filter cigarette C (Table I) was relatively low, we investigated the possibility that 1 was filtered selectively by cellulose acetate filter tips. As is evident from the data in Table II, 1 and 3-ethyl-2-hydroxy-2-cyclopenten-1-one were indeed selectively reduced. The amount of 1 per cigarette was reduced by 91%, while TPM was reduced by only 39%.

To investigate the nature of the precursors to 1 in cigarette smoke, we compared the smoke of some experimental cigarettes (see Table III). The level of 1 in the mainstream smoke of a Bright tobacco cigarette was higher than in the smoke of Oriental or Burley cigarettes. Similar results have recently been reported (Matsushima et al., 1979). Further investigation of Burley cigarettes showed that leaves from the top stalk positions gave smoke with a higher level of 1 (19.6 μg/g) than that observed when leaves from the bottom stalk position were used (3.6 μg/g). Comparison of the levels of 1 in cigarettes made from a standard experimental blend (SEB IV) and from a hexane–ethanol azeotrope extracted SEB IV indicated that the precursors to 1 were extractable under these conditions (see Table III). The concentration of 1 in a nontobacco,

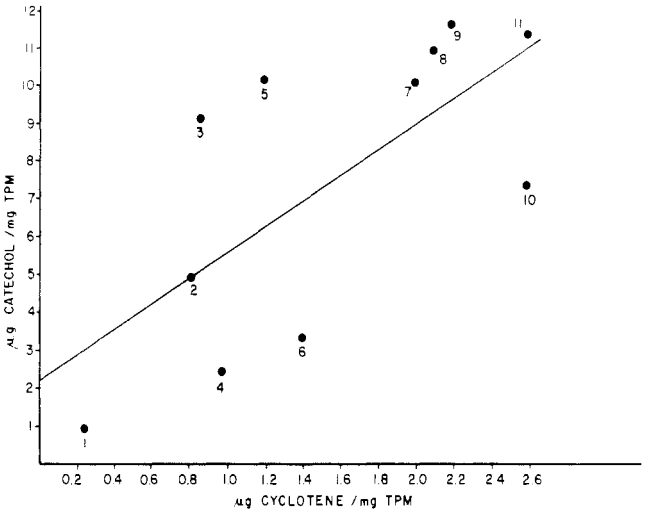


Figure 3. Correlation between levels of catechol and 2-hydroxy-3-methyl-2-cyclopenten-1-one in the smoke of 11 nonfilter cigarettes. Code: (1) Burley, bottom stalk position; (2) Burley, top stalk position; (3) reference IRI (Table III, entry 1); (4) SEB IV, extracted (Table III, entry 6); (5) cigarette B (Table I, entry 2); (6) Burley cigarette (Table III, entry 3); (7) Oriental cigarette (Table III, entry 4); (8) cigarette C without filter (Table II, entry 1); (9) cigarette A (Table I, entry 1); (10) SEB IV (Table III, entry 5); (11) Bright cigarette (Table III, entry 2).

modified cellulose cigarette was only 3.7 μg/cigarette.

The results of these analyses were parallel in certain respects to results obtained when cigarette smoke was analyzed for catechol. Bright tobacco gave smoke with relatively high levels of catechol (Kallianos et al., 1968; Matsushima et al., 1979). Levels of catechol were higher in smoke from the top leaves of Burley tobacco compared to the bottom leaves (Hoffmann et al., 1980), and the concentration of catechol was markedly reduced in the smoke of hexane–ethanol-extracted cigarettes (Brunnemann et al., 1976). We investigated further the possible relationship of smoke catechol and cyclotene levels for several cigarettes as shown in Figure 3. A least-squares regression analysis showed that the correlation between catechol and cyclotene levels was 0.694 ($P < 0.05$). The regression line fit for the data is

$$\text{catechol} = 2.19 + 3.40(\text{cyclotene})$$

These data strongly suggest that catechol and 1 have similar leaf precursors. Further studies are necessary to determine the nature of these precursors.

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Oxidation of Glutathione by Hydrogen Peroxide and Other Oxidizing Agents

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Reduced glutathione (GSH) in aqueous solution was oxidized with hydrogen peroxide, benzoyl peroxide, potassium bromate, and linoleic acid hydroperoxide. The resulting oxidized compounds were measured by amino acid analysis or by ^{1}H nuclear magnetic resonance. Glutathione was oxidized at various pH values, and the ratios of the oxidation products were measured. The various hydrolysis products observed after acid hydrolysis and amino acid analysis are discussed and related to the results with ^{1}H NMR on intact glutathione.

Food proteins are often exposed to oxidants or oxidizing conditions during processing and storage. Oxidizing agents such as benzoyl peroxide and potassium bromate, for example, are added to bread doughs to improve baking quality, and hydrogen peroxide is used to control microorganisms and improve the color of various food products during processing. Although not added deliberately, lipid hydroperoxides, well-known for causing off-flavors and odors in food products, are probably the most common oxidizing agents in foods. They are formed from unsaturated fatty acids by either enzyme or free

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