Saliva-Available Carbonyl Compounds in Some Chewing Tobaccos

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Carbonyl compounds leached from three types of commercial chewing tobaccos were quantitated by derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride, hexane extraction of the *O*-oxime derivatives, and gas chromatography/mass spectrometry relative to authentic standards. The major carbonyl compounds were 5-(hydroxymethyl)furfural (predominant), glyoxal, methylglyoxal, and acetaldehyde, all being mutagens. Other mutagenic compounds or carcinogens detected included formaldehyde, crotonaldehyde, and furfural. These carbonyl compounds did not significantly contribute to the Microtox acute toxicity of the chewing tobacco leachates.

Keywords: *Tobacco; aldehydes; ketones; Microtox, saliva*

Regular chewing tobacco (CT) use can cause oral lesions, leukoplakia, gingival recession, cancer at the site of contact, and also other cancers (U.S. Department of Health and Human Services, 1986; NIH Consensus Development Panel, 1988; Christen, 1992). Tobacco chewing is incorrectly claimed to be a healthy alternative to smoking (Goolsgy, 1992; NIH Consensus Development Panel, 1988). About 75% of 30 300 annual cases of oropharyngeal cancers in the United States has been attributed to smokeless tobacco (ST) usage and about 90% to use of both smokeless and smoking tobacco (Marwick, 1993). These rates may increase since about 20% of U.S. high school males surveyed in 1991 had used CT or snuff during the previous 30 days (McCann, 1993). Taxes are also relatively low for ST products. The publicity about the dangers of smoking has caused many to continue their nicotine habit with ST (Marwick, 1993).

Irritative effects are implicated in the genesis of carcinogenicity at the site of CT contact (U.S. Department of Health and Human Services, 1986). Nicotine is the major irritant leached into artificial saliva from CT; other compounds at concentrations between 10 and 25% that of nicotine were 1*H*-indole-3-acetonitrile, dihydroactinidiolide [5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4*H*)-benzofuranone], and an unidentified compound (Chou and Que Hee, 1994). Other compounds must initiate CT cancer since nicotine is not mutagenic (McCann *et al.*, 1975) or carcinogenic in standard bioassays. Nicotine increases the potencies of carcinogens by membrane damage due to its irritative properties enhancing carcinogen uptake (Squier and Johnson, 1993). The roles in CT oral carcinogenesis of tobacco-specific *N*-nitrosamines, nitrosamino acids, ^{210}Po, benz[*a*]pyrene, and other CT components, though much investigated, have still not been elucidated (U.S. Department of Health and Human Services, 1993; McClennan, 1991; O'Neill *et al.*, 1991). CT contains 0.67–8.2 ppm of *N'*-nitrosonornicotine (NNN), 0.03–3 ppm of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 0.36–7 ppm of *N'*-nitrosoanatabine (NAT) plus *N'*-nitrosoanabasine (NAB); snuff has 0.8–89, 0.1–14, and 0.2–220 ppm, respectively (Hoffmann and Hecht, 1985; Hecht and Hoffmann, 1988).

Formaldehyde, acetaldehyde, furfural, and crotonaldehyde are carcinogenic aldehyde irritants, which are also found in tobacco smoke (NRC, 1986) and ST (Rix *et al.*, 1977; Sharma *et al.*, 1991). Recent National Toxicology Program studies have shown malonaldehyde sodium (National Toxicology Program, 1988) and furfural (National Toxicology Program, 1990) to be carcinogens but not benzaldehyde, 2-chloroacetophenone, and *d*-carvone. Carbonyl compounds are important to tobacco taste and aroma (Weybrew and Stephens, 1962; Stedman, 1968; Weeks *et al.*, 1992) and perhaps aid nicotine habituation. Carbonyl compounds such as syringaldehyde, 4-hydroxyacetophenone, and acetovanillone are also added as flavorants (Brunnemann and Hoffmann, 1993).

The major constituents of CT are Wisconsin and Pennsylvania air-cured tobaccos and Burley tobacco, with smaller amounts of flue-cured "Virginia" (smoking) types. The major carbonyl compounds in the headspaces of five Burley tobaccos (Rix *et al.*, 1977) were isovaleraldehyde (31–60%), *n*-valeraldehyde (13–36%), 2-methylbutyraldehyde (13–22%), and solanone (14–26%) with some benzaldehyde, *trans*-5-methyl-3-hexen-2-one, and 6-methyl-5-hepten-2-one. Similarly, the headspaces of 13 flue-cured tobaccos had (Rix *et al.*, 1977) isovaleraldehyde (4.4–15%), 2-methylbutyraldehyde (2.3–11%), *n*-valeraldehyde (31–67%), 1-hexanal (8.5-17%), and 6-methyl-5-hepten-2-one (13–35%). Headspace analyses for Pennsylvania and Wisconsin tobaccos are not available.

Efforts have been made to identify tobacco compounds that transfer to the vapor/particulate phase during smoking as opposed to being pyrolysis products (Demole and Berthet, 1972; Kimland *et al.*, 1972; Lloyd *et al.*, 1976; Wahlberg *et al.*, 1977; Sakai *et al.*, 1984; Weeks *et al.*, 1989, 1992). Such carbonyl compounds could also transfer into saliva from CT with the addition of the more water soluble nonvolatile compounds.

Analysis of steam-distillable volatiles of 15 flue-cured tobaccos has revealed that carbonyl compounds present in concentrations greater than 5 ppm were damascenone, 3-hydroxy-β-damascone, 4-keto-α-ionol, 5-methylfurfural, solanascone, solanone, and 1,3,7,7-tetramethyl-9-oxo-2-oxabicyclo[4.4.0]dec-5-ene (Weeks *et al.*, 1989). Some of these compounds may arise from thermal

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degradation of flavor additives (LaVoie *et al.*, 1985). Carbonyl compounds detected in the steam distillates from CTs included benzaldehyde, furfural, 5-methylfurfural, and phenylacetaldehyde (LaVoie *et al.*, 1985). The carbonyl compounds found in steam distillation volatiles may also be available to saliva during tobacco chewing except for the pyrolysis and hydrolysis products. The aldehydes that are endogenous or produced by pyrolysis and hydrolysis during steam distillation have not been differentiated.

The major aim of the present study was to determine the identities and quantities of endogenous carbonyl compounds in CT available to artificial saliva in a system that simulated chewing.

MATERIALS AND METHODS

Rationale. The CT chosen were the most popular in the United States (Siegel *et al.*, 1992). An artificial simulated saliva [Chou and Que Hee, 1994, modified after Spector (1956) and Tenovuo (1989)] was a reference extraction medium to eliminate the notorious biological variabilities of human salivas and to aid cross-comparisons between investigators. Chewing was simulated by choosing an average contact time (Hatsukami *et al.*, 1988, 1991) at body temperature. Carbonyl compounds were quantified by reaction with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) and then gas chromatography/mass spectrometry (GC/MS) of the extracted *O*-oximes (Glaze *et al.*, 1989; Cancilla and Que Hee, 1992; Cancilla *et al.*, 1992).

Reagents. Three brands of CT, all in 3 oz packs, were purchased: Redman long cut chewing tobacco (RM; Owensboro, KY), Beech-Nut wintergreen chewing tobacco (BN; Louisville, KY), and Levi Garett chewing tobacco (LG; Winston-Salem, NC). Artificial pH 7.0 simulated human saliva was the extraction medium. It comprised 1400 mg of sodium chloride/L, 500 mg of potassium/L as potassium chloride, 100 mg of calcium/L as calcium chloride, and 150 mg of phosphorus/L as sodium dihydrogen phosphate, all from Fisher Scientific. The medium also contained 25 mg of magnesium/L as magnesium chloride, 2700 mg of mucin type III/L, 88 mg of urea/L, 200 mg of glucose/L, 100 units of amylase/mL, 700 units of lysozyme/L, and 4 units of phosphatase/L, all from Sigma Chemical. Hydrochloric acid, sulfuric acid, and sodium hydroxide (all for pH adjustment), OPTIMA hexane (extractions), and nitric acid (glassware cleaning) were from Fisher Scientific.

Microtox test bioassay reagents, described elsewhere (Chou and Que Hee, 1992, 1993, 1994), were from Microbics Corp. PFBHA and internal standards, decafluorobiphenyl (DFB) and 1,2-dibromopropane (DBP), were from Aldrich Chemical. Aldehydes [acetaldehyde, *n*-propanal, *n*-butanal, *n*-pentanal, *n*-heptanal, *n*-decanal, isobutyraldehyde, acrolein, crotonaldehyde, 2-furfural (furfural), *trans*-2-hexenal, 2-acetylpyrrole, 5-(hydroxymethyl)furfural, benzaldehyde, methylglyoxal, and glyoxal] and ketones (acetone, 2-butanone, 2-heptanone, 2,4-hexanedione, 1-decalone, heptanophenone, ionone, 2-methylcyclohexanone, acetophenone, 6-methyl-5-hepten-2-one, and 4-hydroxy-5-methyl-4-cyclopentene-1,3-dione) were from Aldrich except formaldehyde (Fisher Scientific).

Apparatus. A shaking water bath (Fisher Scientific, Model 125, no. 429) extracted CT powder into saliva. A vortex mixer (Thermolyne, Type 16700 mixer, Dubuque, IA) aided organic extractions. An Accumet pH meter Model 825MP (Fisher Scientific) measured pH. Other apparatus such as the Microtox test system, software (revised Microtox 6.0), computer, nylon filters, centrifuge, coffee milling machine, and screw-cap test tubes have been described (Chou and Que Hee, 1994). A Hewlett-Packard 5890A gas chromatograph 5988A/mass spectrometer utilized a 30 m × 0.317 mm i.d. DB-1701 1.0 μm 14% cyanopropylphenyl bonded stationary phase fused-silica capillary column (J&W Scientific). The electron multiplier operated in the total ion current mode (TIC) from 30 to 550 amu for the 70 eV electron impact source at 300 °C.

Methods. *CT Extraction*. The CT was ground in the coffee milling machine for 30 s. The milled CT was still wet so it could not be sieved to assess particle size distribution. When dry, the particle size ranged from 18 to 450 μm. Artificial saliva (6 mL) was mixed with 2 g of wet CT in an 8 mL Kimax test tube by vortex for 30 s and shaken in a water bath for 1 h at 37.0 ± 0.2 °C and 60 rpm to simulate oral temperature and mastication. The solution was centrifuged at 3400 rpm for 15 min at room temperature. The liquid phase was transferred by Pasteur pipet for filtration followed by rinsing with 1 mL of artificial saliva. The rinsate was then combined with the aliquot before filtration through nylon filters of 5.0, 1.2, and 0.45 μm pore size in that order. The pH and the volume of the filtered extract (the leachate) were measured. One milliliter was evaporated in a stream of nitrogen and the residue dried to constant weight in a vacuum desiccator. A saliva control was also processed in parallel.

Carbonyl Compound Analysis in CT Extracts. The method is modified from one described elsewhere (Cancilla *et al.*, 1992). A volume of 0.1 mL of 0.1 N sodium thiosulfate was added to 5 mL of leachate with shaking. The pH was adjusted to 1.1 with HCl and then 0.5 mL of 10 mg/mL PFBHA added. After shaking, the samples were microwaved for 20 s to a temperature of about 80 °C (bubbles just appear) and then cooled by standing at room temperature, and finally 50 μL of 18 N sulfuric acid was added with shaking. Three extractions with hexane (1 mL) followed, with amalgamation of all hexane extracts in one vial. The hexane solution was concentrated 6-fold. A 40-fold concentration allowed identification. Anhydrous sodium sulfate (50 mg) was added and the sample shaken. A 2 μL volume of 0.56 mg/mL DBP as internal standard was added, and 4.6 μL of the hexane solution was injected for TIC GC/MS analysis using the splitless mode with a purge delay of 0.75 min and an injector temperature of 250 °C. Helium was the carrier gas at 3 mL/min. The temperature program was 50 °C for 6 min, 5 °C/min to 250 °C, and 250 °C for 4 min. The transfer line temperature was 275 °C. Identification was by comparison with mass spectra of *O*-oxime standards and their retention times.

Microtox Testing. Toxic compounds cause a decrease of luminescence in the bioluminescent bacterium *Photobacterium phosphoreum*. The concentration to diminish the light emission by half at a given challenge time, the EC_{50}, is a measure of acute toxicity (Chou and Que Hee, 1992, 1993, 1994). Microtox tests for the leachates and for the corresponding aqueous layer after hexane extraction of PFBHA-derivatized carbonyl compounds were performed at optimal conditions including readjustment of the pH to 6.5 as described elsewhere (Figure 1) to produce EC_{50} values at 5, 15, and 25 min (Chou and Que Hee, 1993). Color correction was necessary except for the saliva control and for PFBHA.

RESULTS AND DISCUSSION

The respective pH and the dissolved solids concentrations were as follows: RM, 5.7 ± 0.2 and 172 ± 8 mg/mL; BN, 5.4 ± 0.2 and 178 ± 5 mg/mL; LG, 5.8 ± 0.2 and 165 ±5 mg/mL; control, 5.9 ± 0.1 and 5.5 ± 0.3 mg/mL. The dissolved solids for leachates did not differ at $p < 0.05$. The arithmetic mean/standard deviation for the three tobaccos was 171.6 ± 6.5 mg/mL with an average weight of leached CT of 166.1 ± 6.8 mg/mL. Since the average leachate volume was 4.45 mL, the average mass leached was 741 mg or 37 ± 2% of the original 2 g of CT.

Table 1 presents the concentrations of the carbonyl compounds >250 ng in injected PFBHA derivative for the leachates, equivalent to about 9 ppm before hexane extraction. The limits of quantification for all carbonyl compounds in the leachates are 100−1000 ppb depending on molecular weight. 5-(Hydroxymethyl)furfural was of high concentration (>7 ppm) (58 ppm for the LG leachate) in all three CT and was 24−87% of the carbonyl compounds leached. Glyoxal (810−8400 ppb),

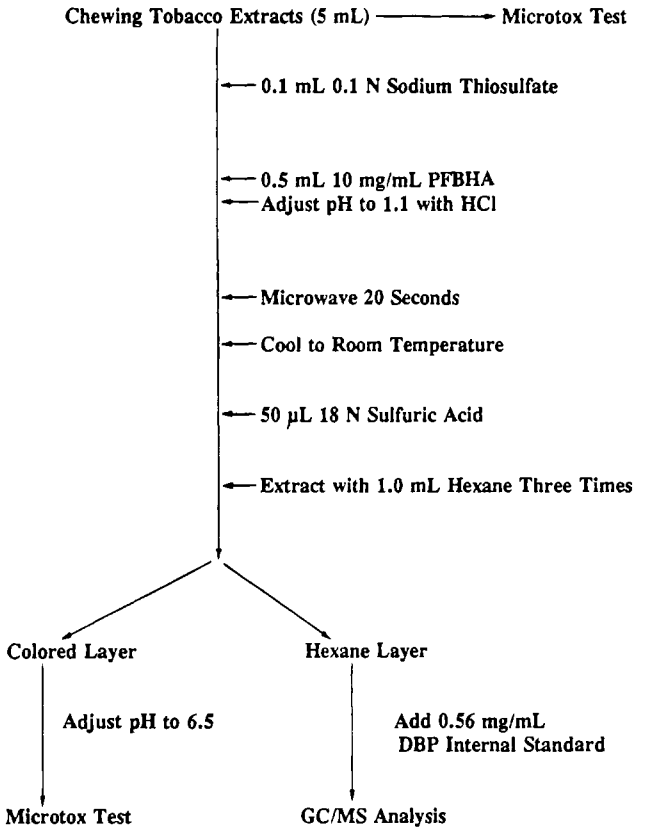

Figure 1. PFBHA [*O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride) derivatization for GC/MS and Microtox analysis scheme for chewing tobacco leachates. DBP is 1,2-dibromopropane, internal standard; and GC/MS is gas chromatography/mass spectrometry.

Table 1. Concentrations of Carbonyl Compounds in Artificial Saliva Filtrate (SF) and Leachates from Chewing Tobaccos (RM, Redman; BN, Beech-Nut; LG, Levi Garett)[a]

carbonyl compd	SF	RM[b]	BN[b]	LG[b]
acetaldehyde	9.0	1500	1600	460
acetone	49	260	210	240
acrolein	nd[c]	60	1400	620
benzaldehyde	nd	*d*	320	*d*
crotonaldehyde	nd	160	1400	*d*
formaldehyde	nd	110	670	530
2-furfural	nd	150	*d*	500
glyoxal	70	2000	8400	810
2-heptanone	nd	580	600	2100
5-(hydroxymethyl)furfural	250	7900	7500	58000
methylglyoxal	250	1500	7600	720
valeraldehyde	nd	640	1300	2300
total		15000	31000	67000

[a] Data are in ng/mL; all coefficients of variation are <10%. [b] Data were corrected for SF and utilized both *E* and *Z* isomers. [c] Not detected (about 10 ng of injected mass of PFBHA–carbonyl derivative). [d] Trace (between detection limit and reporting threshold of 250 ng in a 4.6 μL injection).

methylglyoxal (720–7600 ppb), 2-heptanone (580–2100), and *n*-valeraldehyde (640–2300 ppb) were >580 ppb in all CT. The carcinogens formaldehyde (112–670 ppb), acetaldehyde (460–1560 ppb), crotonaldehyde (trace–1400 ppb), and furfural (trace–500 ppb) were also present. The only other major toxic carbonyl was acrolein in BN and LG.

The *Salmonella* mutagens were furfural, glyoxal, and methylglyoxal (Kier *et al.*, 1986; Lewis, 1991). The only detected nonmutagen was acetone (Kier *et al.*, 1986). 5-(Hydroxymethyl)furfural is a mutagen (Shahabuddin *et al.*, 1991) present in foods, beverages, bacterial cultures, and hydrothermalized wood (Cook *et al.*, 1989) and is a thermal degradation product of sugars containing glucose. It has been detected in flue-cured tobacco (Stedman, 1968; Lloyd *et al.*, 1976) but not in CT headspaces. It is postulated to react with adenine–thymine base pairs in duplex DNA (Shahabuddin *et al.*, 1991) like the carcinogen furfural (National Toxicology Program, 1990).

Table 1 results differ from those for steam distillation analysis of CT volatiles during which pyrolysis and hydrolysis also occur. Carbonyl compounds reported previously were benzaldehyde, furfural, 5-methylfurfural, and phenylacetaldehyde (LaVoie *et al.*, 1985). The leachates of the present study contained benzaldehyde and furfural. 5-(Hydroxymethyl)furfural is a near relative of 5-methylfurfural. La Voie *et al.* (1985) linked the furfural derivatives to thermal decomposition of sugar additives or the tobacco pectin. The latter is unlikely since only the soluble components of the leachates were derivatized, not the whole CT; the heating step was very short and at low temperature.

Analysis of steam-distillable volatiles of 15 flue-cured tobaccos showed carbonyl compounds in concentrations greater than 5 ppm for damascenone, 3-hydroxy-β-damascone, 4-keto-α-ionol, 5-methylfurfural, solanascone, solanone, and 1,3,7,7-tetramethyl-9-oxo-2-oxabicyclo-[4.4.0]dec-5-ene (Weeks *et al.*, 1989). Carbonyl compounds found in most varieties were 5-methylfurfural and solanone, neither of which was found in the present study. Headspace analysis of volatiles of flue-cured tobaccos provides another perspective. Rix *et al.* (1977) reported large amounts of *n*-valeraldehyde and 6-methyl-5-hepten-2-one with smaller amounts of isovaleraldehyde, 2-methylbutyraldehyde, and *n*-hexanal. Similarly, Burley tobacco headspace had mostly isovaleraldehyde followed by *n*-valeraldehyde, 2-methylbutyraldehyde, and then solanone in that order. Green uncured bright leaf tobacco headspace contained isovaleraldehyde, 3-pentanone, *n*-hexanal, *trans*-2-hexenal, benzaldehyde, and 6-methyl-5-hepten-2-one. The assignments were made assuming equal MS response factors; no concentrations were actually reported. In the present study, *n*-valeraldehyde was important but solanone was not because of solubility.

In all types of tobacco, acetaldehyde dominates (63–79% w/w) for the lower aliphatic carbonyls excluding formaldehyde up to *n*-valeraldehyde (Weybrew and Stephens, 1962). Acetone was next in content (11–20% w/w). Acetaldehyde was always greater than acetone in the leachates of the present CT study.

Table 2 gives the mass spectra of PFBOA–oxime derivatives of some aldehydes and ketones potentially present in CT. Those for acetophenone, 2-acetylpyrrole, acrolein, isobutyraldehyde, crotonaldehyde, furfural, 2-heptanone, heptanophenone, 2,4-hexanedione, 4-hydroxy-5-methyl-4-cyclopentane-1,3-dione, 5-(hydroxymethyl)furfural, ionone, methyl ethyl ketone, 6-methyl-5-hepten-2-one, and 2-methylcyclohexanone are published for the first time. The base ion except for 2-acetylpyrrole, 2,4-hexanedione, and ionone was *m/z* 181. The identity of 5-(hydroxymethyl)furfural was obtained by retention times and mass spectra, compared with those for other M^+ of 321. PFBHA–6-methyl-5-hepten-2-one had a retention time (32 min) that was much shorter than that of 5-(hydroxymethyl)furfural (43 min). The retention time of PFBHA–4-hydroxy-5-methyl-4-cyclopentene-1,3-dione was near that of 5-(hy-

Table 2. Mass Spectra of PFBHA–Oxime Derivatives of Some Aldehydes and Ketones in Order of Retention Time

compd	M+[a]	RT[b]	mass to charge ratio (intensity)
formaldehyde	225	16.499	181 (100), 195 (8.6), 182 (7.6), 161 (6.6), 117 (5.0), 167 (3.7), 99 (3.6), 93 (2.9)
acetaldehyde	239	19.654	181 (100), 209 (8.4), 182 (7.4), 161 (4.6), 117 (3.2), 167 (2.6), 195 (2.5), 99 (2.3)
		19.909	
acetone	253	21.494	181 (100), 182 (7.2), 72 (5.8), 223 (4.6), 253 (4.5), 206 (4.5), 236 (3.6), 161 (3.5)
acrolein	251	22.800	181 (100), 182 (8.4), 251 (5.8), 250 (5.7), 161 (5.2), 117 (3.3), 221 (3.8), 195 (2.8)
		23.300	
propionaldehyde	253	22.835	181 (100), 236 (8.3), 182 (7.5), 223 (3.3), 161 (3.2), 195 (2.7), 117 (2.0), 167 (1.9)
		23.042	
isobutyraldehyde	267	23.477	181 (100), 195 (9.3), 182 (6.7), 250 (6.2), 43 (6.1), 99 (3.5), 93 (3.1), 161 (2.8)
methyl ethyl ketone	267	23.843	181 (100), 56 (35.0), 250 (12.1), 58 (9.6), 86 (7.7), 182 (7.3), 195 (6.3), 55 (4.2), 161 (3.2), 43 (2.6)
n-butyraldehyde	267	24.779	181 (100), 239 (11.3), 182 (7.5), 195 (5.2), 250 (4.9), 41 (3.5)
		24.995	
crotonaldehyde	265	27.358	181 (100), 250 (20.6), 182 (6.9), 43 (6.9), 39 (6.5), 161 (4.0), 265 (3.6), 117 (3.0)
		27.688	
valeraldehyde	281	27.884	181 (100), 239 (14.4), 182 (7.5), 41 (6.5), 161 (3.3), 207 (3.2), 39 (3.0), 195 (2.9)
		28.015	
2-heptanone	309	30.343	181 (100), 72 (29), 253 (28), 41 (10.8), 55 (10.5), 177 (8.6), 42 (8.4), 128 (7.7)
		30.716	
furfural	291	31.26	181 (100), 291 (21.1), 83 (11.3), 248 (9.7), 52 (7.7), 182 (7.6), 39 (6.9), 80 (6.2)
		31.929	
trans-2-hexenal	293	31.472	181 (100), 250 (15.4), 182 (7.8), 71 (7.3), 112 (5.1), 53 (4.2), 54 (4.1), 161 (2.9)
		32.018	
n-heptaldehyde	309	32.178	181 (100), 239 (17), 182 (7.5), 41 (5.8), 43 (5.2), 207 (5.2), 128 (4.0), 55 (3.8)
		32.289	
6-methyl-5-hepten-2-one	321	32.483	181 (100), 69 (95.0), 83 (92.0), 82 (85.8), 55 (38.8), 67 (20.8), 108 (18.0), 72 (15.4)
		32.934	
2-methylcyclohexanone	307	32.709	181 (100), 96 (90.2), 126 (54.8), 81 (48.6), 67 (38.6), 95 (25.8), 55 (18.4), 41 (16.4)
2,4-hexanedione	309	33.228	57 (100), 181 (28.7), 112 (6.9), 72 (6.7), 252 (6.1), 58 (3.4), 182 (2.0), 161 (1.7)
		33.417	
		33.569	
		34.003	
acetophenone	315	35.443	181 (100), 315 (30.8), 314 (30.3), 77 (30), 106 (17.6), 103 (15.6), 78 (14), 65 (14.5)
		37.889	
benzaldehyde	301	36.462	181 (100), 301 (13.2), 77 (8.6), 182 (7.5), 65 (7.5), 271 (7.4), 89 (5.8), 51 (5.2)
2-acetylpyrrole	304	37.625	93 (100), 304 (80.3), 82 (70.3), 181 (29.3), 92 (22.9), 66 (15.7), 123 (14.2), 305 (11.3)
		38.059	
n-decanal	351	38.779	181 (100), 239 (21), 41 (7.6), 43 (7.3), 182 (7.1), 55 (5.4), 69 (4.7), 170 (4.2)
4-hydroxy-5-methyl-4-cyclopentene-1,3-dione	321	40.936	181 (100), 321 (13.8), 110 (10.1), 82 (10.0), 182 (7.4), 53 (5.1), 55 (3.9), 83 (3.0)
1-decalone	347	41.017	181 (100), 166 (93.7), 79 (74.5), 67 (64.4), 93 (40.5), 55 (37.0), 81 (36.2), 91 (35.6)
		41.184	
methylglyoxal	462	41.291	181 (100), 182 (7.1), 265 (4.9), 161 (3.0), 195 (2.3), 167 (1.9), 117 (1.4), 99 (1.1)
		41.938	
		42.432	
glyoxal	448	41.860	181 (100), 182 (7.3), 161 (3.3), 195 (2.6), 448 (2.3), 167 (2.1), 117 (1.7), 99 (1.3)
		41.979	
		42.131	
5-(hydroxymethyl)furfural	321	42.825	181 (100), 79 (29.2), 123 (25.8), 321 (23.9), 110 (11.5), 113 (10.6), 81 (8.9), 278 (6.1)
		43.007	
heptanophenone	385	43.024	181 (100), 104 (85.8), 77 (81.5), 103 (58.9), 134 (54.0), 91 (54.0), 119 (46.7), 315 (41.3)
		44.313	
ionone	387	43.190	372 (100), 206 (47.7), 181 (47.3), 91 (37.8), 174 (36.4), 160 (33.6), 55 (26.5), 105 (26.5)
		43.858	

[a] Molecular ion mass to charge ratio. [b] Retention time in minutes. The different RT are for the *E* and *Z* geometric isomers which have identical mass spectra. Both were utilized for quantitative purposes.

droxymethyl)furfural, but the intensities of *m/z* 321 and 278 did not match.

Non-carbonyl compounds are also present, coextracted at the acidic conditions. Peaks at retention times of 23.6 and 26.5 min were 2,4-hexadienedioic (muconic) and benzoic acids; and peaks at 14.7 and 20.8 min were solvent contaminants. An unidentified compound at 20.8 min coeluted with DFB internal standard in many samples. DBP (retention time 10.3 min) was therefore used instead. Unreacted PFBHA was also present in the hexane extract after derivatization, signifying quantitative reaction (Jehlar *et al.*, 1994). The acidic extraction conditions caused nicotine to be in the quaternary salt form that is very water soluble and hence not extractable by hexane. This eliminated the potentially serious interferences of the alkaloids during chromatography as many of them are at much higher concentrations than carbonyls.

The Microtox EC_{50} values for PFBHA were 0.95 ± 0.03 mg/mL at 5 min, 0.88 ± 0.03 mg/mL at 15 min, and 0.90 ± 0.02 mg/mL at 25 min. This is the first report of the Microtox EC_{50} value for PFBHA. Since PFBHA scavenges carbonyl compounds, it will also scavenge the natural luciferin, *n*-tetradecanal, and so a low EC_{50} value might be expected. This experiment was performed to assess if the excess PFBHA after derivatization had to be removed by addition of sulfuric acid or not before Microtox analysis of the aqueous residue after hexane extraction. Figure 2 gives the EC_{50} (expressed as percentage recovered of the original CT leachate soluble dissolved solids) of leachates before and after PFBHA derivatization. EC_{50} values after PFBHA derivatization gave significant statistical differences at 5 and 15 min at $p < 0.05$ but not at 25 min. There were no differences at $p < 0.01$. Thus, analysis at 25 min is recommended. This implied carbonyl compounds in leachates do not contribute significant Microtox toxicity, this being confirmed by their low concentrations and supporting our previous conclusion that nicotine is the major available irritant (Chou and Que Hee, 1994).

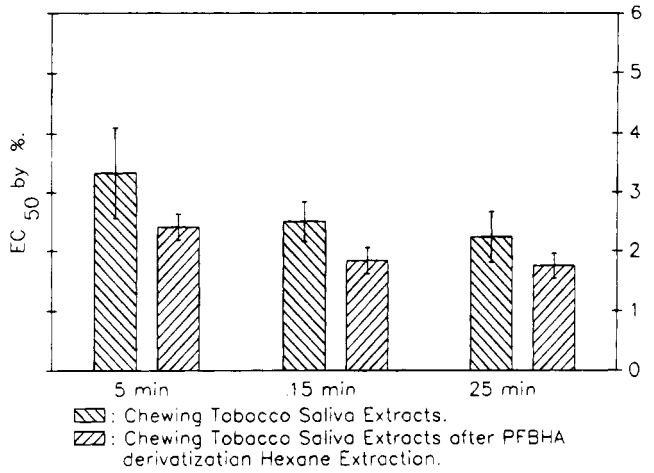

Figure 2. Comparison of EC_{50} values of leachate of RM (Redman) chewing tobacco and of aqueous solution after PFBHA derivatization–extraction.

The contributions of carbonyl compounds to mutagenicity or carcinogenicity may be very different from that for acute toxicity and require further investigation.

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