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Short communication

Quantitative analysis by gas chromatography of volatile carbonyl compounds in cigarette smoke

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Abstract

The cigarette smoke from 26 commercial brands was drawn into a separatory funnel containing an aqueous cysteamine solution. Almost the entire smoke from a cigarette was trapped as mainstream cigarette smoke. The carbonyl compounds in the smoke were derivatized to thiazolidines and were then quantitatively analyzed by gas chromatography with nitrogen–phosphorus detection. Total carbonyl compounds recovered ranged from 2.37 to 5.14 mg/cigarette. The general decreasing order of the carbonyl compounds yielded was acetaldehyde, butanal, hexanal, propanal, acetone, octanal, 2-methylpropanal and formaldehyde. Acetaldehyde was the major aldehyde in the smoke sample from 26 brands and it made up 46–72% of the total carbonyl compounds in the sample. Amounts of formaldehyde ranged from 73.8 to 283.8 $\mu\text{g}/\text{cigarette}$. It is hypothesized that these carbonyl compounds form from lipid and wax constituents in tobacco leaves.

1. Introduction

In the last three decades, tremendous numbers of chemicals have been isolated and identified in cigarette smoke as a result of the refinement of advanced analytical instruments such as the gas chromatograph–mass spectrometer. Consequently, the presence of many toxic chemicals such as polynuclear aromatic hydrocarbons [1], N-nitrosamines [2] and volatile aldehydes [3,4] have been identified in cigarette smoke. These chemicals in smoke are deposited directly into the blood following inhalation. In contrast to benzo[*a*]pyrene and N-nitrosornicotine which require enzyme activation to be toxic [5], reactive

aldehydes such as formaldehyde and acetaldehyde can directly cross-link to proteins and bind covalently to nucleic acids [6–8], and consequently cause biological complications including carcinogenesis [9–12]. For example, formaldehyde reportedly induced squamous cell carcinoma in the nasal cavity of rats upon repeated inhalation [13]. Acetaldehyde is also capable of inducing nasal carcinomas in experimental animals [14].

The quantitation of these toxic aldehydes in cigarette smoke is of great importance because tobacco smoking is one of the major causes of aldehyde contamination of indoor air [15]. For example, formaldehyde in sidestream cigarette smoke can mean considerable exposure for the non-smoker through passive smoking [16]. According to the technical support document pre-

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pared by the California Environmental Protection Agency/Air Resources Board [17], ambient acetaldehyde is an air pollutant which may cause or contribute to an increase in mortality or an increase in serious illness, or which may pose a present or potential hazard to human health based on the findings of carcinogenicity and the results of the risk assessment.

Some highly volatile or reactive compounds such as formaldehyde, acetaldehyde and acrolein are still extremely difficult to analyze. There are, therefore, only a few reports on direct analysis of these reactive carbonyls in cigarette smoke [18]. There are also very few reports on accurate quantitative analysis of volatile carbonyl compounds in cigarette smoke. Most commonly used analytical methods for volatile carbonyl compounds involve derivatization with 2,4-dinitrophenylhydrazine (2,4-DNP) and the resulting derivatives are determined by GC or HPLC. After the 2,4-DNP method was used to determine formaldehyde in cigarette smoke in the 1960s [19], many volatile carbonyl compounds have been identified in cigarette smoke using this method. For example, formaldehyde (25–69 $\mu\text{g}/\text{cigarette}$) and acetaldehyde (752–1234 $\mu\text{g}/\text{cigarette}$) were reported in mainstream cigarette smoke [20].

We recently developed a simple and sensitive analytical method to identify volatile carbonyl compounds in foods and beverages [21,22]. This method involves derivatization of carbonyl compounds with cysteamine to yield stable thiazolidines. The resulting thiazolidines are determined by GC with a fused-silica capillary column and nitrogen–phosphorus detection (NPD). Cysteamine readily reacts with carbonyl compounds at room temperature and neutral pH. The only drawback of this method is that cysteamine does not react with α,β -unsaturated aldehydes such as acrolein. Vapor-phase formaldehyde was satisfactorily analyzed using this derivatization method [23]. In the present study, vapor-phase carbonyl compounds formed in the cigarette smoke from various commercial brands were quantitatively analyzed using the above method.

2. Experimental

2.1. Materials

All cigarette samples were bought from local markets and stored in sealed packages until used. Cysteamine hydrochloride, thiazolidine and 2,4,5-trimethylthiazole were purchased from Aldrich, Milwaukee, WI, USA. The standard stock solution of 2,4,5-trimethylthiazole was prepared by adding 10 mg of 2,4,5-trimethylthiazole to 1 ml of chloroform; it was stored at 5°C. The authentic thiazolidines were synthesized according to the method reported previously [22,24].

2.2. Sample preparations

Cysteamine hydrochloride (0.8 g) was dissolved in 200 ml of deionized water and the pH of the solution was adjusted to 8 with a 6 M NaOH solution. The cysteamine solution (approximately 200 ml) was placed in a 1000-ml separatory funnel whose headspace was evacuated at 8.4 mmHg (1 mmHg = 133.322 Pa) for 10 min. Immediately after a cigarette was lit, about 1 mm of the other end of the cigarette was inserted in the tip of the separatory funnel. Most cigarettes were fitted into the tip of the funnel. Cigarettes with a diameter smaller than the inside diameter of the tip of the funnel were wrapped with masking tape in order to fit. The cock of the separatory funnel was opened gradually to draw the smoke into the separatory funnel. It took 20 s to completely smoke one cigarette. After smoke was sucked into the separatory funnel, the funnel was shaken for 5 min in order to let the carbonyl compounds in the smoke react thoroughly with the cysteamine. After the pH of the reaction mixture was adjusted to 7 with a 1 M HCl solution, it was extracted with 50 ml of chloroform using a liquid–liquid continuous extractor for 3 h. The extract was dried over anhydrous sodium sulfate for 12 h. After removal of the sodium sulfate, the volume of the extract was adjusted to exactly 50 ml with chloroform. A standard solution of 2,4,5-trimethylthiazole (100 μl) was added as an

internal standard prior to GC analysis. An aqueous solution (200 ml) containing 0.8 g of cysteamine was extracted with 50 ml chloroform at pH 8, the same as for the smoke samples, and the extract was used as a blank sample.

2.3. Instrumental analysis

A Hewlett-Packard (HP) Model 5890A GC system equipped with a NPD system and a 30 m \times 0.25 mm I.D. (film thickness, $d_f = 1 \mu\text{m}$) DB-1 bonded phase fused-silica capillary column was used for quantitative analysis of thiazolidines. The oven temperature was programmed from 60 to 180°C at 4°C/min and held for 10 min. Peak areas were integrated with a Tsp SP 4400 series integrator. The injector and detector temperatures were 250°C. The linear velocity of helium carrier gas was 30 cm/s. The quantitative analysis was conducted according to the internal standard method previously reported [25].

A HP Model 5890 series II GC system interfaced to a HP 5971 mass spectrometer was used to confirm the thiazolidine derivatives in the samples. The GC conditions were as above. The mass spectra were obtained by electron impact ionization at 70 eV and an ion source temperature of 250°C.

3. Results and discussion

The optimum yield of each thiazolidine from the reaction of a corresponding aldehyde and cysteamine was previously obtained at pH 8 in our laboratory [26]. In the present study, the recovery efficiencies of the carbonyl compounds from an aqueous solution were 92.8% for formaldehyde, 96.0% for acetaldehyde, 98.2% for acetone, 95.3% for propanal, 93.0% for 2-methylpropanal, 92.3% for butanal, 88.0% for hexanal and 99.8% for octanal (the values are the mean of four replications). The NPD detection limits of each aldehyde were 5.8 pg for formaldehyde, 6.9 pg for acetone, 7.1 pg for acetaldehyde, 10.0 pg for propanal, 12.5 pg for

2-methylpropanal, 14.4 pg for butanal, 24.7 pg for hexanal and 36.5 pg for octanal.

As mentioned above, the most commonly used derivative for analysis of volatile carbonyl compounds is 2,4-DNP. There are several HPLC methods for the separation of 2,4-DNP derivatives obtained from carbonyl compounds present in cigarette smoke [20,27,28]. However, HPLC analysis of the 2,4-DNP derivatives is difficult because they produce *syn*- and *anti*-forms except in the case of formaldehyde. Moreover, preparation of 2,4-DNP derivatives requires a strong acidic condition which may alter the chemicals of interest. In contrast to 2,4-DNP preparation, carbonyl compounds readily react with cysteamine at neutral pH and room temperature.

Many different kinds of smoking machines which are constructed to simulate human smoking have been used for the study of cigarette smoke [29]. The amounts of smoke collected in the present study are not similar to those inhaled

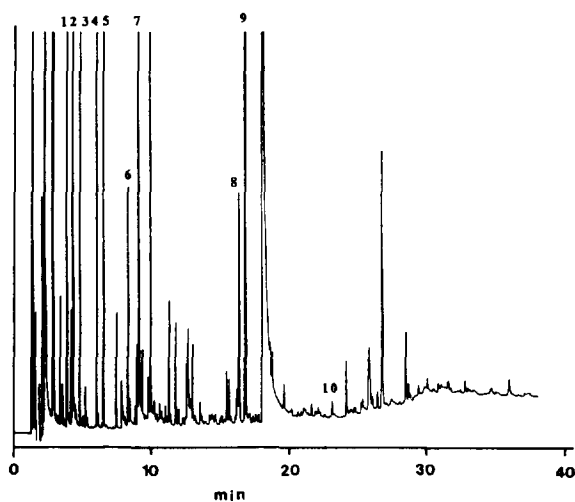


Fig. 1. A typical gas chromatogram of the extract from cigarette smoke (cigarette N) trapped in a cysteamine solution. Peaks: 1 = thiazolidine (formaldehyde); 2 = 2-methylthiazolidine (acetaldehyde); 3 = 2,2-dimethylthiazolidine (acetone); 4 = 2,4,5-trimethylthiazole (internal standard); 5 = 2-ethylthiazolidine (propanal); 6 = 2-isopropylthiazolidine (2-methylpropanal); 7 = 2-propylthiazolidine (butanal); 8 = 2-pentylthiazolidine (hexanal); 9 = (S)-(-)-nicotine; 10 = 2-heptylthiazolidine (octylaldehyde).

by a smoker because a smoking machine was not used. All of the smoke trapped in the present study might be classified as mainstream cigarette smoke.

Fig. 1 shows a typical GC–NPD separation of the chloroform extract from the cigarette smoke trapped in a cysteamine solution. All peaks in this chromatogram contain one or more nitrogen atoms. Table 1 presents the results of quantitative analysis of volatile carbonyl compounds. The greatest and the least amounts of total carbonyl compounds obtained were 5.135 and 2.365 mg/cigarette, respectively. There was no

significant difference in aldehyde formation in menthol and non-menthol cigarettes. With a few exceptions, the general decreasing order of each aldehyde yielded was acetaldehyde, butanal, hexanal, propanal, acetone, octanal, 2-methylpropanal and formaldehyde. For example, the amount of formaldehyde (237.5 μg) recovered was the second largest (after acetaldehyde) in the case of one cigarette.

Acetaldehyde was the major aldehyde in the smoke from all brands analyzed in the present study. Acetaldehyde as a percentage of the total carbonyl compounds ranged from 49.1 to 72.1.

Table 1

Amounts of formaldehyde (FA), acetaldehyde (AA), acetone, propanal (PA), 2-methylpropanal (2-MPA), butanal (BA), hexanal (HA) and octanal (OA) yielded in the smoke from various brands of cigarettes

Cigarette brand	Amounts ($\mu\text{g}/\text{cigarette}$)							
	FA	AA	Acetone	PA	2-MPA	BA	HA	OA
A ^{a,b}	101.4 \pm 10.0	2154 \pm 280.7	206.7 \pm 70.5	245.5 \pm 53.4	111.6 \pm 18.5	441.0 \pm 66.4	234.0 \pm 51.8	166.7 \pm 47.8
B ^{a,b}	265.7 \pm 33.9	2109 \pm 119.7	188.9 \pm 31.4	256.6 \pm 36.5	100.2 \pm 17.8	374.2 \pm 43.6	331.1 \pm 52.8	288.3 \pm 56.5
C ^{a,b}	109.2 \pm 12.0	2024 \pm 105.1	239.3 \pm 41.7	250.0 \pm 33.1	122.3 \pm 16.6	462.4 \pm 66.7	380.9 \pm 45.0	194.2 \pm 53.2
D ^a	176.7 \pm 23.7	2274 \pm 62.6	206.7 \pm 46.3	245.6 \pm 23.1	92.4 \pm 9.1	175.4 \pm 31.1	112.2 \pm 55.4	82.0 \pm 38.4
E ^a	157.5 \pm 5.4	2815 \pm 11.9	559.0 \pm 21.9	349.3 \pm 17.4	147.7 \pm 5.2	601.9 \pm 40.7	289.5 \pm 48.5	177.3 \pm 42.4
F ^a	79.1 \pm 6.9	1722 \pm 34.0	154.4 \pm 39.9	198.0 \pm 24.8	80.2 \pm 6.8	166.7 \pm 41.9	157.1 \pm 42.8	87.4 \pm 45.1
G ^a	237.5 \pm 7.8	1924 \pm 28.1	203.3 \pm 29.9	228.3 \pm 21.7	90.8 \pm 7.3	319.6 \pm 29.7	180.1 \pm 61.2	148.9 \pm 64.3
H ^a	138.8 \pm 11.5	2221 \pm 253.4	236.6 \pm 45.5	236.1 \pm 48.5	97.4 \pm 16.6	539.5 \pm 1.9	165.0 \pm 67.1	165.3 \pm 46.1
I ^a	73.8 \pm 5.0	2030 \pm 130.6	191.3 \pm 41.0	233.8 \pm 42.2	105.0 \pm 20.5	155.3 \pm 49.4	180.5 \pm 64.0	101.7 \pm 51.5
J ^a	87.6 \pm 8.0	2245 \pm 46.4	395.3 \pm 7.7	257.1 \pm 18.2	110.3 \pm 5.0	296.5 \pm 24.0	179.1 \pm 49.4	97.6 \pm 30.6
K ^a	123.9 \pm 18.5	2166 \pm 107.0	233.4 \pm 34.5	253.0 \pm 37.0	103.0 \pm 17.5	395.1 \pm 32.1	190.7 \pm 48.5	76.1 \pm 35.7
L ^a	84.6 \pm 15.8	1706 \pm 15.6	132.7 \pm 23.9	186.0 \pm 27.1	68.5 \pm 10.8	88.6 \pm 13.0	102.1 \pm 56.9	–
M ^d	118.6 \pm 10.2	2099 \pm 12.7	136.8 \pm 46.7	207.0 \pm 59.8	77.9 \pm 19.3	236.5 \pm 39.4	144.2 \pm 75.5	55.2 \pm 39.1
N ^{a,c}	143.8 \pm 20.5	2377 \pm 35.2	249.2 \pm 110.5	226.7 \pm 89.7	81.5 \pm 32.3	433.0 \pm 145.1	209.4 \pm 74.5	76.0 \pm 39.6
O ^{a,c}	115.7 \pm 9.6	2015 \pm 103.1	246.7 \pm 35.4	242.0 \pm 41.8	98.2 \pm 12.2	287.8 \pm 47.0	225.8 \pm 62.2	120.4 \pm 28.3
P ^d	119.4 \pm 9.5	2902 \pm 12.0	278.5 \pm 30.0	316.3 \pm 21.2	133.1 \pm 5.5	429.2 \pm 28.1	314.0 \pm 48.9	157.0 \pm 32.8
Q ^d	206.1 \pm 17.3	2706 \pm 63.8	283.4 \pm 75.0	298.3 \pm 50.3	132.2 \pm 17.3	553.8 \pm 161.6	297.4 \pm 84.4	217.3 \pm 34.0
R ^d	183.3 \pm 5.6	2591 \pm 30.6	255.9 \pm 18.1	304.6 \pm 18.0	122.2 \pm 3.7	787.7 \pm 12.1	368.1 \pm 42.0	258.2 \pm 93.4
S ^d	107.2 \pm 16.7	1990 \pm 43.5	202.3 \pm 17.9	230.8 \pm 17.6	92.8 \pm 4.0	119.9 \pm 17.8	133.6 \pm 42.7	–
T ^d	125.2 \pm 6.2	2705 \pm 33.9	619.4 \pm 17.6	336.2 \pm 34.4	140.9 \pm 12.1	514.1 \pm 25.8	144.2 \pm 35.9	124.5 \pm 31.9
U ^d	125.7 \pm 13.2	2356 \pm 174.6	210.4 \pm 32.8	275.9 \pm 31.7	114.2 \pm 5.9	440.8 \pm 100.2	300.5 \pm 67.4	187.1 \pm 67.0
V ^d	122.8 \pm 7.4	1909 \pm 52.9	153.7 \pm 19.2	217.6 \pm 28.0	87.5 \pm 7.6	142.8 \pm 13.9	124.9 \pm 32.7	–
W ^{d,c}	123.3 \pm 10.1	2403 \pm 46.5	220.8 \pm 17.4	268.6 \pm 16.2	105.9 \pm 3.9	115.6 \pm 16.8	137.4 \pm 47.5	98.5 \pm 44.1
X ^c	205.6 \pm 8.7	2315 \pm 59.1	312.8 \pm 20.9	284.7 \pm 20.9	115.9 \pm 3.1	519.0 \pm 43.7	261.9 \pm 55.8	197.0 \pm 70.6
Y ^c	151.3 \pm 10.1	1491 \pm 177.1	142.1 \pm 42.1	171.1 \pm 41.3	76.3 \pm 15.7	413.7 \pm 60.1	212.1 \pm 58.5	128.2 \pm 35.6
Z ^c	283.8 \pm 26.2	2521 \pm 78.6	312.7 \pm 61.9	302.0 \pm 22.8	115.0 \pm 10.9	928.3 \pm 149.5	369.9 \pm 74.4	308.3 \pm 103.3

^a Regular size.

^b No filter.

^c Menthol.

^d King size.

^e Super king size.

The amounts of acetaldehyde recovered ranged from 1.5 to 2.9 mg. Butanal was found in large amounts in the smoke from some cigarettes. For example, it composed 18% of the smoke in the case of one cigarette.

The precursors and formation mechanisms of these volatile carbonyl compounds in the cigarette smoke are not yet well understood. However, it is well known that these carbonyl compounds are formed from lipids by heat treatment [30–33]. Tobacco contains certain amounts of lipids [34] and waxes [35] which may be precursors of these carbonyl compounds. Aldehydes and ketones have been reportedly formed from heat treatment of various lipids via oxidative cleavage of the double bond; examples include beef fats [3,31], pork fat [32] and corn oil [33]. Oxidative degradation of squalene isolated from tobacco smoke [36] also yielded formaldehyde, acetaldehyde, and acetone [37]. It was proposed that many low-molecular-mass radicals such as $\cdot\text{OH}$, $\cdot\text{CHO}$, $\cdot\text{CH}_2\text{CHO}$, $\cdot\text{CH}_3$, and $\cdot\text{COCH}_3$ were formed from lipids upon oxidative degradation and that these radicals combine to form low-molecular-mass aldehydes [38]. Presence of radicals has been recognized in the cigarette smoke by using an electron spin resonance spectrometer [39].

4. Conclusions

The sample collection method developed in the present study does not require a large, heavy trapping device. Once flasks are vacuumed, they can be carried to any place to collect smoke samples in order to monitor toxic carbonyl compounds contamination in the ambient air. The analytical method used for volatile carbonyl compounds in cigarette smoke in the present study is simple, highly sensitive, and specific. Also, baseline separation of all thiazolidine derivatives was obtained with a high resolution fused-silica capillary column. The cigarette smoke trapped in the present study is not comparable to that trapped using a cigarette machine. Comparison of the levels of carbonyl compounds found in the present study to the

previous studies is not within the scope of this study.

References

- [1] *The Evaluation of the Carcinogenic Risk to Humans: Tobacco Smoking (IARC Monographs, Vol. 38)*, International Agency for Research on Cancer, Lyon, 1986, pp. 168–170.
- [2] S.S. Hecht, C.-H.B. Chen, G.D. McCoy and D. Hoffmann, in J.-P. Anselme (Editor), *N-Nitrosamines (ACS Symposium Series, No. 101)*, American Chemical Society, Washington, DC, 1986, pp. 125–152.
- [3] *Environmental Health Criteria 89*, World Health Organization, Geneva, 1989.
- [4] E.J. Bardana, Jr. and A. Montanaro, *Ann. Allergy*, 66 (1991) 441.
- [5] T. Sugimura, *Cancer*, 49 (1982) 1970.
- [6] C. Auerbach, M. Moutschen-Dahman and J. Moutschen, *Mutat. Res.*, 39 (1977) 317.
- [7] A.J. Fornace, Jr., J.F. Lechner, R.C. Graftstrom and C.C. Harris, *Carcinogenesis*, 3 (1982) 1373.
- [8] C.W. Lam, M. Casanova and H.D. Heck, *Fundam. Appl. Toxicol.*, 6 (1986) 541.
- [9] A.K. Basu, L.J. Marnett and L.J. Romano, *Mutat. Res.*, 129 (1984) 39.
- [10] R.C. Graftstrom, A. Fornace, Jr. and C.C. Harris, *Cancer Res.*, 44 (1984) 4323.
- [11] B.M. Goldschmidt, *J. Environ. Sci. Health*, C2 (1984) 231.
- [12] V. Nair, C.S. Cooper, D.E. Vietti and G.A. Turner, *Lipids*, 21 (1986) 6.
- [13] R.E. Albert, A.R. Sellakumar, S. Laskin, M. Juschner, N. Nelson and C.A. Snyder, *J. Natl. Cancer Inst.*, 68 (1982) 597.
- [14] Y.J. Feron, A. Kruyssen and R.A. Woutersen, *Eur. J. Cancer Clin. Oncol.*, 18 (1982) 13.
- [15] S.E. Feinman (Editor), *Formaldehyde Sensitivity and Toxicity*. CRC Press, Boca Raton, FL, 1988.
- [16] *Formaldehyde and Other Aldehydes*. National Research Council, National Academy of Sciences, Washington, DC, 1980.
- [17] *Acetaldehyde as a Toxic Air Contaminant*, California Environmental Protection Agency, Air Resources Board, Stationary Source Division, Sacramento, CA, 1993.
- [18] K.D. Brunnemann, M.R. Kagan, J.E. Cox and D. Hoffmann, *Carcinogenesis*, 11 (1990) 1863.
- [19] R.E. Leonard and J.E. Kiefer, *J. Gas Chromatogr.*, 4 (1966) 142.
- [20] P.R. Houlgate, K.S. Dhingra, S.J. Nash and W.H. Evans, *Analyst*, 114 (1989) 355.
- [21] T. Hayashi, C.A. Reece and T. Shibamoto, *J. Assoc. Off. Anal. Chem.*, 69 (1986) 101.

- [22] T. Miyake and T. Shibamoto, *J. Agric. Food Chem.*, 41 (1993) 1968.
- [23] A. Yasuhara and T. Shibamoto, *J. Assoc. Off. Anal. Chem.* 72 (1989) 899.
- [24] A. Yasuhara and T. Shibamoto, *Agric. Biol. Chem.*, 53 (1989) 2273.
- [25] L.S. Ettre, in L.S. Ettre and A. Zlatkis (Editors), *The Practice of Gas Chromatography*. Interscience, New York, 1967, p. 402.
- [26] A. Yasuhara and T. Shibamoto, *J. Chromatogr.*, 547 (1991) 291.
- [27] D.L. Manning, M.P. Maskarinec, R.A. Jenkins and A.H. Marshall, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 8.
- [28] C.T. Mansfield, B.T. Hodge, R.B. Hege and W.C. Hamlin, *J. Chromatogr. Sci.*, 15 (1977) 301.
- [29] E.L. Wynder and D. Hoffmann, *Tobacco and Tobacco Smoke*, Academic Press, New York, 1967, pp. 1–2.
- [30] S. Ohnishi and T. Shibamoto, *J. Agric. Food Chem.*, 32 (1984) 987.
- [31] K. Umano and T. Shibamoto, *J. Agric. Food Chem.*, 35 (1987) 14.
- [32] A. Yasuhara and T. Shibamoto, *J. Food Sci.*, 54 (1989) 1471.
- [33] C. Macku and T. Shibamoto, *J. Agric. Food Chem.*, 39 (1991) 1265.
- [34] M.K. Wassef and J.W. Hendrix, in *Proceedings of the University of Kentucky Tobacco and Health Workshop Conference, March 1973*. Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, 1973, pp. 768–773.
- [35] W. Carruthers and R.A.W. Johnstone, *Nature*, 184 (1959) 1131.
- [36] A. Rodgman, L.C. Cook and C.K. Chappell, *Tobacco Sci.*, 5 (1961) 1.
- [37] H.C.H. Yeo and T. Shibamoto, *Lipids*, 27 (1992) 50.
- [38] F. Niyati-Shirhodaee and T. Shibamoto, *J. Agric. Food Chem.*, 41 (1993) 227.
- [39] J.R. Wasson, H.E. Francis, G.L. Seebach and L. de Salles de Hys, in *Proceedings of the University of Kentucky Tobacco and Health Workshop Conference, March 1973*, Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, 1973, pp. 474–484.