that complain of unexplained symptoms following extremely low-level FA inhalation.

7. Conclusion

Long-term exposure to low levels of FA can disturb normal homeostatic responses by enhancing a neural network that is abnormally activated by coexposure to immunological stimuli and results in the induction of neurogenic and immunogenic inflammation in the brain. These responses may be related to the triggering of MCS. Possible target organs and biomarkers affected by formaldehyde exposure with or without OVA were shown in Figure 1.

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Simultaneous determination of volatile organic compounds and carbonyls in mainstream cigarette smoke using a sorbent cartridge followed by two-step elution

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ABSTRACT

We developed a simple method for the simultaneous determination of volatile organic compounds (VOCs) and carbonyls in the mainstream cigarette smoke using a sorbent cartridge at ambient temperature without the traditional cryogenic impinger. A sorbent cartridge is installed between intake filter and the pump of the smoking machine. Collection of cigarette mainstream smoke is performed according to the Canadian Intense regime or the ISO regime. As adsorbent, Carboxen 572 (CX-572) is the most suitable for collection of VOCs and carbonyls in the mainstream cigarette smoke. Elution of VOCs and carbonyls from CX-572 is performed by the two-step elution with carbon disulfide and methanol. VOCs are eluted by first elution with carbon disulfide and carbonyls are eluted by second elution with methanol. For VOCs, a portion of eluate is analyzed by gas chromatography–mass spectrometry. For carbonyls, a portion of eluate is derivatized with enriched 2,4-dinitrophenylhydrazine solution and analyzed by high-performance liquid chromatography. Measurement values by CX-572 cartridge method are very close to those obtained by traditional impinger method except for 2-butanone. Impinger methods use 2,4-dinitrophenylhydrazine solution containing 50% water and 2-butanone-DNPhydrazone may be hydrolyzed with water. In the CX-572 method, the hydrolysis of 2-butanone is prevented because the eluate solution contains no water. CX-572 method can measure cigarette smoke resulting from not only one whole cigarette but also from one puff volume because of its high sensitivity and simple operation.

1. Introduction

Cigarette smoking is associated with a variety of pulmonary and cardiovascular disorders including emphysema, atherosclerosis and cancer [1–5], and causes 30% of all cancer deaths. Cigarette smoke, which can be divided into gas phase and particulate matter, is a complex mixture consisting of more than 5000 chemicals and at least 50 of these are carcinogenic [6,7]. Volatile organic compounds (VOCs) in gas phase cigarette smoke include hazardous substances, specifically, benzene, 1,3-butadiene, isoprene and acrylonitrile that are carcinogenic and prevalent toxins [8,9]. Benzene induces leukemia both in humans with occupational exposures [10,11] and in experimental animals [12,13]. In International Agency for Research on Cancer (IARC) monographs [14], benzene was classified as a Group-1 compound (*carcinogenic to humans*), citing additional evidence of an increased incidence of acute nonlymphocytic leukemia in workers exposed to benzene. 1,3-Butadiene is known to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in humans, including epidemiological and mechanistic studies [15]. Similarly to benzene, 1,3-butadiene was currently classified as a Group-1 carcinogen by IARC [16]. Isoprene has been identified as a carcinogen in humans and experimental animals [17] and was classified as a Group 2B carcinogen (*possibly carcinogenic to humans*) on the basis of sufficient evidence for carcinogenicity at multiple organ sites in both mice and rats, especially male mice, exposed by inhalation [18]. Acrylonitrile was found to be carcinogenic to rats with tumors reported in the central nervous system, mammary gland, and a few other rare tumor sites. IARC classified acrylonitrile in 1999 as Group 2B, based on evidence from experimental animals [19]. Similarly to VOCs, carbonyls such as aldehydes and ketones in cigarette smoke, have received much attention as hazardous substances in studies of environmental and biological chemistry. Long-term exposure to relatively high levels of formaldehyde is known to increase the risk to humans [20–23]. In 2004, IARC reclassified formaldehyde as a Group 1 human carcinogen that causes nasopharyngeal cancer and also concluded that there is a "strong but not sufficient evidence for a causal association between leukemia and occupational exposure to formaldehyde" [24]. Acetaldehyde may be responsible

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for increased risk of head and neck cancer and esophageal cancer and was classified as a Group 2B substance [25–27]. Acrolein is not currently a suspected human carcinogen as, to date, no studies have been conducted to observe its carcinogenic effects on human cells. However, studies in rats have shown an increase in cancerous tumors from ingestion but not inhalation, and Feng et al. [28] have recently suggested a connection between acrolein in cigarette smoke and an increased risk of lung cancer.

Therefore, it is very important to measure VOCs and carbonyls in cigarette smoke and evaluate the effect of smoking on human health. At the present time, measurement of VOCs and carbonyls in the mainstream cigarette smoke is performed by two different collection methods and two analytical instruments. In the VOCs analysis, generally, measurement is performed under Health Canada Intense Regime (HCI) T-116 [29] or CORESTA Recommended Method (CRM) No. 74 [30]. In these regulations, VOCs are collected by passing the mainstream smoke of two or five cigarettes through a glass fiber filter disk and into the cryogenic impinger containing 10 mL methanol cooled to below −70 °C by using dry-ice/isopropanol bath. Then, the impinger solutions are injected onto a gas chromatograph/mass spectrometer (GC/MS) for quantitation. In the carbonyl analysis, generally, measurements are performed under HCI T-104 [31] or CRM No. 70 [32]. In these regulations, carbonyls are collected by passing the mainstream smoke of two or five cigarettes into the impinger containing 80 mL or 35 mL DNPH solution. Then, the impinger solutions are injected onto a high performance liquid chromatography (HPLC) for quantitation.

Thus, these traditional impinger methods need large sampling devices, cryogenic bath cooled to below −70 °C for VOCs, and very complicated operation. Therefore, we developed the simple method for the measurement of VOCs in the mainstream cigarette smoke using a sorbent cartridge at ambient temperature without the cryogenic impinger.

2. Experimental

2.1. Apparatus and reagents

The GC/MS system (QP 2010 Ultra, Shimadzu, Kyoto, Japan) was used with a fused-silica column (InertCap AQUATIC-2 60 m × 0.25 mm i.d., d = 1.4 μm, GL Sciences, Tokyo, Japan) and operated with temperature programming from 40 °C (held for 6 min) to 250 °C at 6 °C/min, with He as carrier gas at 0.61 mL/min and 70 eV EIMS detection operated in full-scan mode from m/z 40–500. The injection volume is 1 μL (split injection, split ratio 10:1; septum purge 1 mL/min; injector temperature 240 °C. The HPLC system (Prominence LC-20, Shimadzu, Kyoto, Japan) was used with two LC-20AD pumps, an SIL-20AC autosampler and an SPD M20A photo-diode array detector. The analytical column was an Ascentis RP-Amide, 3 μm particle size, 150 mm × 3 mm i.d. column (Supelco Inc., Bellefonte, PA, USA). Solution A of the mobile phase mixture was acetonitrile/water (50/50, v/v) containing 10 mmol/L ammonium acetate and solution B was acetonitrile/water (80/20, v/v). HPLC elution was carried out with 100% A for 5 min, followed by a linear gradient from 100% A to 100% B in 50 min and then held for 10 min. The flow rate of the mobile phase was 0.7 mL/min. The column temperature was 30 °C and the injection volume was 10 μL.

The cigarette smoke was generated on a single-port piston-type smoking machine Model LM1/PLUS (Heinrich Borgwaldt Hamburg, Germany). Thermal mass flow meter (TSI 4100 Series, TSI Inc.) was used for measuring the smoking machine puff profiles.

Standard 1,3-butadiene solution (2.0 mg/mL in methanol) was purchased from AccuStandard Inc. (New Haven, CT, USA). Benzene-d6 (99.95%), Isoprene (95.0%), acrylonitrile (97%) benzene (99.7%), toluene (99.7%), carbon disulfide (99.0%) and methanol (99.8%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Carboxen 563 (CX-563, 20/45 mesh), Carboxen 564 (CX-564, 20/45 mesh), Carboxen 569 (CX-569, 20/45 mesh), Carboxen 572 (CX-572, 20/45 mesh) and activated carbon (AC, 20/45 mesh) were purchased from Sigma–Aldrich Inc. (MO, USA). Anasorb 747 (AS-747, 20/40 mesh) was purchased from SKC Inc. (PA, USA). The water used for HPLC and sample preparation was deionized and purified using a Milli-Q Water System equipped with a UV lamp (Millipore, Bedford, MA, USA). 2,4-Dinitrophenylhydrazine hydrochloride (>98%) was obtained from Tokyo Kasei Co., Ltd. (Tokyo, Japan). The acetonitrile (HPLC grade, >99.9%), ethanol (>99.5), phosphoric acid (85% solution in water), and ammonium acetate (99.999%) were from Sigma–Aldrich Inc. (MO, USA).

Sample cigarettes used in this study were 3R4F, 1R5F from University of Kentucky (Lexington, KY, USA) and CM6 from Cooperation Center for Scientific Research Relative to Tobacco (CORESTA, Paris, France).

2.2. Preparation of the sorbent cartridge (CX-572 cartridge)

CX-572 particles were weighed into a glass tube and conditioned at 380 °C for 5 h under a flow of purified nitrogen at 50 mL/min using a tube conditioner (TC-20, Markes Int. Ltd., Mid-Glamorgan, UK). After cooling to room temperature, carbon adsorbents were packed into the polyethylene cartridges (Rezorian tube, 1 mL, Supelco Inc., Bellefonte, PA).

2.3. Preparation of the enriched DNPH-solution

Phosphoric acid (25 mL) and DNPH hydrochloride (1 g) are added in a 50 mL volumetric flask, and then this solution is diluted to 50 mL with acetonitrile. This mixture solution is continuously stirred with a magnetic stirrer until a clear solution is obtained and stored in a refrigerator at 4 °C.

2.4. Collection cigarette smoke using a CX-572 cartridge

Test cigarettes are placed at 22 °C temperature and 60% humidity. CX-572 cartridge is installed between intake filter and pump of the smoking machine. Collection of cigarette main-stream smoke is performed according to the HCI regime [33] or the ISO regime [34]. In the HCI regime, mainstream smoke constituents are collected under the conditions of 55 mL puff volume, 2 s puff duration, 30 s puff interval, and 100% blocking of the filter ventilation holes with Mylar adhesive tape. In the ISO regime, mainstream smoke constituents are collected under the conditions of 35 mL puff volume, 2 s puff duration, 60 s puff interval, and no blocking of the filter ventilation holes.

2.5. Elution of CX-572 cartridge by two-step elution and analysis

After collection, CX-572 particles together with the frits are removed from cartridge and deposited into the 15 mL septum-sealed vial. Then, 1 mL of carbon disulfide is slowly added into the vial through the septum using the syringe with needle. After letting the sample stand for 10 min, 4 mL of methanol is added and stirred for 10 s. In the case of VOC analysis, a 1 mL portion of eluate solution is transferred to a 1.5 mL autosampler vial, internal standard (10 mg/mL benzene-d6, 8 μL) is added, and then, this solution is analyzed by GC/MS under the conditions described in Section 2.1. In the case of carbonyl analysis, a 0.5 mL of portion of eluate solution is transferred to a 5 mL volumetric flask. Then, 0.1 mL of the enriched DNPH solution is added, and after ten minutes, this solution is diluted to 5 mL with ethanol and analyzed by HPLC.

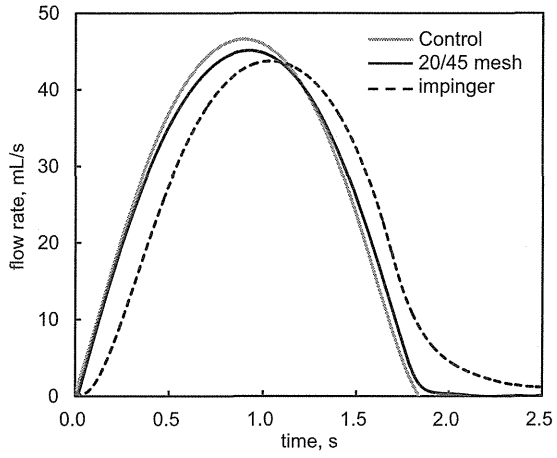

Fig. 1. The changes in flow rates with time during one puff cycle.

3. Results and discussion

3.1. Selection of particle size of adsorbent

Changes in flow rate for puff duration (puff profile) will affect the generation of chemical substances from cigarette. Puff profile of smoking machine may vary depending on particle size of adsorbent in the sorbent cartridge. Therefore, the changes in flow rate and pressure drop at puff duration were measured using a thermal mass flow meter. The test particle sizes of adsorbent were 20/45, 45/60, 60/80, 80/120 mesh. 300 mg of each adsorbent was packed into the polyethylene cartridge. For reference, impinger filled with 35 mL DNPH solution [32] was tested. Thermal mass flow meter (TSI 4100) was set in the smoking machine between cigarette and sorbent cartridge. Then, the smoking machine was run under the conditions of 55 mL puff volume, 2 s puff duration based on the HCI regime [33]. Fig. 1 shows the changes in flow rates with time during one puff cycle. Maximum flow rate was decreased when the particle size was decreased down to 80/120 mesh. The puff duration was longer when particle size decreased. Moreover, pressure drops were observed in cases of smaller particle sizes (data not shown). Puff profile of impinger was shifted toward longer puff duration and not completed within 2 s. Puff profile using 20/45 mesh size is very similar to that with no cartridge (control) and pressure drop is lowest in comparison to that for other mesh sizes. From these results, 20/45 mesh size was selected for the sorbent cartridge.

3.2. Selection of adsorbents for the sorbent cartridge

CX-563, CX-564, CX-569, CX-572, AC, and AS-747 were packed in a two-bed sorbent tube separated with frits (1 mm thickness). Both beds contained the same sorbent. Adsorbent contents of front (F) and rear (R) beds was as follows: F/R; 50 mg/450 mg, 100 mg/400 mg, 200 mg/300 mg, 300 mg/200 mg, 400 mg/100 mg, 500 mg/0 mg. Mainstream cigarette smoke from reference cigarette CM6 was collected with each sorbent tube using smoking machine according to the HCI regime [33]. CM6 generates the highest concentrations of VOCs and carbonyls in mainstream cigarette smoke among the reference cigarettes. After collection, in each case adsorbent particles of front bed and rear bed were removed from sorbent tube and analyzed by the procedure described above. Fig. 2 shows changes in collection efficiency of propene, 1,3-butadiene, isoprene, benzene and toluene with amount of adsorbent. The collection efficiency was calculated from the ratio of the peak area of adsorbates of front (F) and rear (R) beds; $F/(F+R) \times 100$. 500 mg of adsorbents can collect all target compounds except for propene. Propene in cigarette smoke is not a target compound but can become an index component for ability of adsorbents because of its low boiling point (−47.6 °C) and its difficulty to adsorption. Overall adsorption abilities decrease in the following order: CX-572 > AC > AS-747 > CX-564, CX-563, CX-569 and are about the same as the following order of surface area: CX-572 (1100 mg/m^2) > AC (1070 mg/m^2) > AS-747 (980 mg/m^2) > CX-564 (400 mg/m^2), CX-563 (510 mg/m^2), CX-569 (485 mg/m^2).

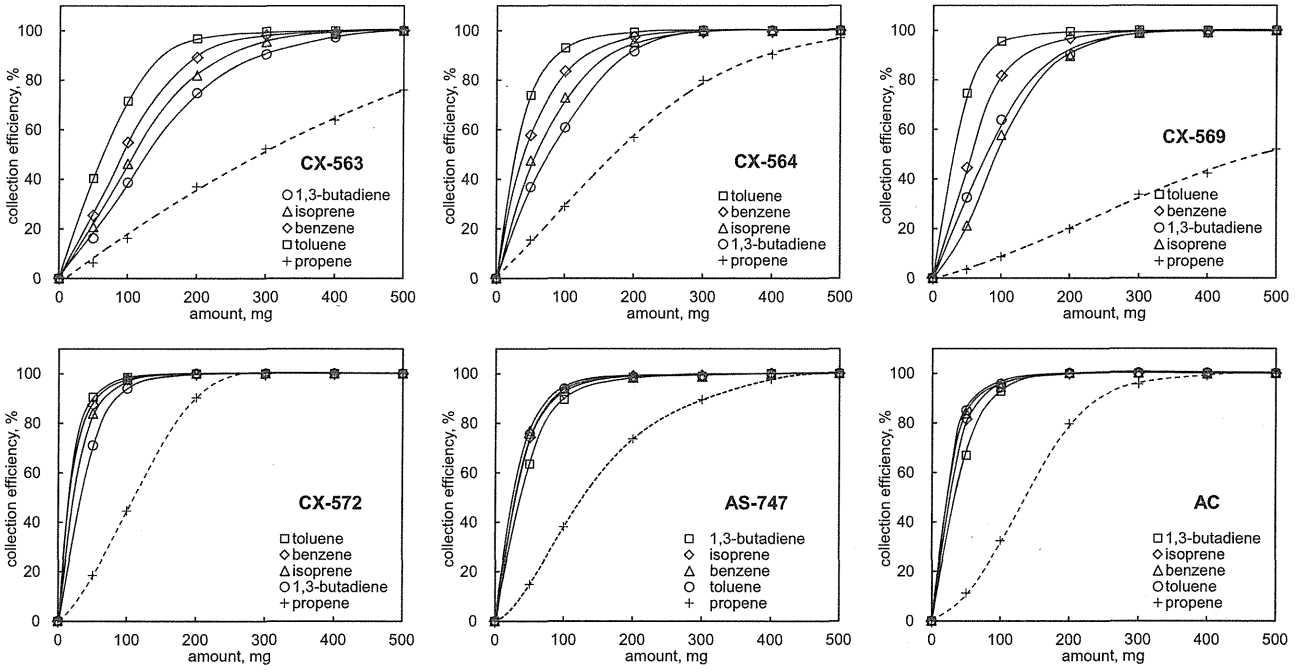

Fig. 2. Changes in collection efficiency with amount of sorbent.

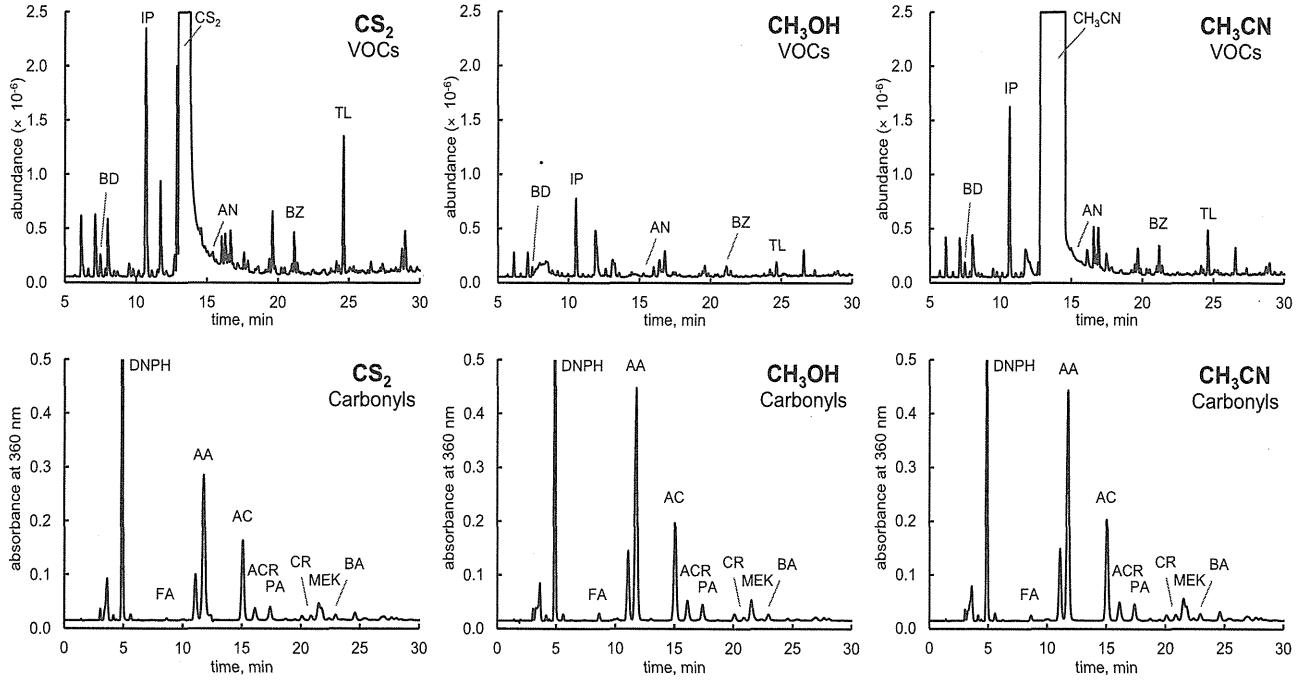

Fig. 3. Chromatographic profiles of VOCs (upper panel) and carbonyls (lower panel) eluted with the various eluates from CX-572 cartridge collected with mainstream cigarette smoke. BD: 1,3-butadiene; IP: isoprene; AN: acrylonitrile; BZ: benzene; TL: toluene; FA: formaldehyde-DNPhydrazone; AA: acetaldehyde-DNPhydrazone; AC: acetone-DNPhydrazone; ACR: acrolein-DNPhydrazone; PA: propanal-DNPhydrazone; CR: crotonaldehyde-DNPhydrazone; MEK: methyl ethyl ketone (2-butanone)-DNPhydrazone; BA: butanal-DNPhydrazone.

Collection efficiencies for carbonyls were similar to VOCs and decrease in the following order: acrolein > acetone > formaldehyde > acetaldehyde. 200 mg of CX-572 completely adsorbed all carbonyls.

Based on these results, 300 mg of CX-572 particles was selected as the sorbent cartridge.

3.3. Selection of eluents for the sorbent cartridge

Mainstream cigarette smoke from reference cigarette CM6 was collected with CX-572 cartridge using a smoking machine according to the HCI regime [33]. After collection, CX-572 particles were removed from cartridge and deposited into the 15 mL septum-sealed vial. Then, 5 mL of methanol, isopropanol, ethyl acetate, acetonitrile, or carbon disulfide was added to the vial using the syringe with needle and the vial was left standing for 30 min. In the case of VOC analysis, a portion of eluate solution was transferred into a 1.5 mL autosampler vial and analyzed by GC/MS under the conditions described above. In the case of carbonyl analysis, a 0.5 mL of portion of eluate solution was transferred to a 5 mL volumetric flask. Then, 0.1 mL of the enriched DNPH solution was added, and after ten minutes, this solution was diluted to 5 mL with ethanol and analyzed by HPLC. Fig. 3 shows chromatograms of VOCs and carbonyls eluted with carbon disulfide, methanol and acetonitrile.

Regarding VOCs, among all the solvents tested, carbon disulfide exhibits excellent elution ability to VOCs, however, cannot completely elute acrylonitrile. Carbon disulfide has week polarity and not suitable for polar substances such as acrylonitrile and carbonyls. Regarding carbonyls, polar solvents such as acetonitrile, methanol, 2-propanol, ethyl acetate and dimethyl sulfoxide exhibit excellent elution ability and can completely elute the target compounds, however, carbon disulfide is not completely to elute carbonyls. Therefore, there were no one solvent acceptable as eluent for both VOCs and carbonyls. Accordingly, the two-step elution with polar and nonpolar solvents was examined. VOCs are eluted by first elution with carbon disulfide and carbonyls are eluted by second elution with methanol. Mainstream cigarette smoke of CM6 was collected with CX-572 cartridge and VOCs and carbonyls were analyzed by the two-step elution method described in Section 2.5. Fig. 4 shows chromatograms of VOCs (upper panel) and carbonyls (lower panel) eluted by the two-step elution method using carbon disulfide and methanol. All target compounds, 1,3-butadiene, isoprene, acrylonitrile, benzene, toluene, acetaldehyde, acetone, acrolein, propanal, 2-butanone and butanal, were completely eluted by the two-step elution method. Moreover, VOCs and carbonyls in eluate solution never readsorb into CX-572 particles in the carbon disulfide/methanol mixture solution.

In the analysis of acrolein using a traditional DNPH-cartridge, acrolein DNPhydrazone is decomposed rapidly in the DNPH-cartridge and forms DNPH and acrolein DNPhydrazone adducts [35–42]. However, CX-572 method uses no DNPH-cartridge and derivatization with DNPH is performed in the eluate solution. When derivatization of carbonyls in the eluate is performed using enriched DNPH solution, acrolein DNPhydrazone decompose in acidified aprotic solvent solution such as acetonitrile, and this decomposition can be inhibited by the addition of a protic solvent such as ethanol [42]. Fig. 5 shows the changes in acrolein DNPhydrazone with time in ethanol, methanol and acetonitrile dilution to the 0.5 mL carbon disulfide – methanol eluate from CX-572 cartridge collected with mainstream cigarette smoke. The decomposition of acrolein DNPhydrazone was depressed by ethanol.

Based on these results, carbon disulfide and methanol were selected as eluents for two-step elution to CX-572 method and ethanol was selected as dilution solvent for carbonyl analysis.

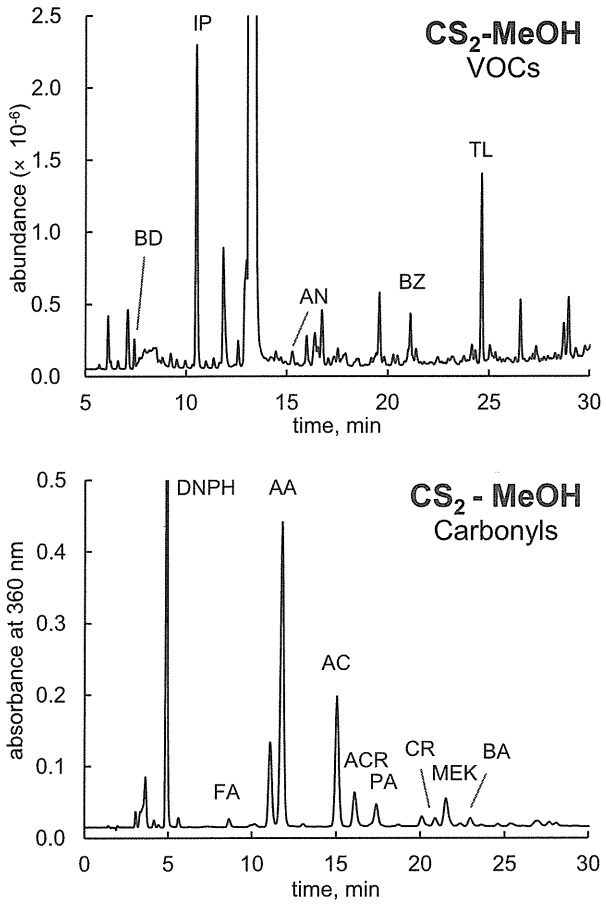

Fig. 4. Chromatographic profiles of VOCs (upper panel) and carbonyls (lower panel) eluted by the two-step elution method using carbon disulfide and methanol. BD: 1,3-butadiene; IP: isoprene; AN: acrylonitrile; BZ: benzene; TL: toluene; FA: formaldehyde-DNPhydrazone; AA: acetaldehyde-DNPhydrazone; AC: acetone-DNPhydrazone; ACR: acrolein-DNPhydrazone; PA: propanal-DNPhydrazone; CR: crotonaldehyde-DNPhydrazone; MEK: methyl ethyl ketone (2-butanone)-DNPhydrazone; BA: butanal-DNPhydrazone.

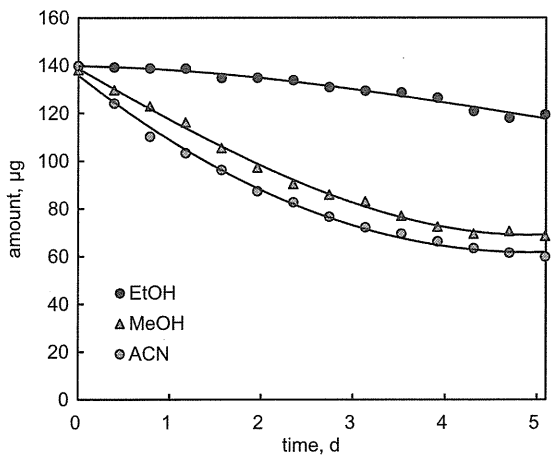

Fig. 5. Decrease of acrolein DNPhydrazone with time in ethanol, methanol and acetonitrile to the eluate from Carboxen 572 cartridge collected with main stream cigarette smoke.

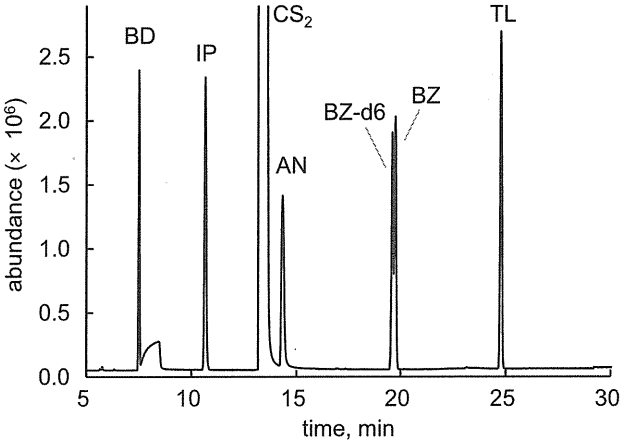

Fig. 6. Total ion chromatogram of a mixed standard solution. BD: 1,3-butadiene; IP: isoprene; AN: acrylonitrile; BZ-d6: benzene-*d*6; BZ: benzene; TL: toluene.

3.4. Preparation of standard solution

The standard stock solutions (2 mg/mL) of isoprene, acrylonitrile, benzene and toluene were prepared by dissolving their reagents in absolute methanol. 1,3-Butadiene is inconvenient for laboratory use because it has very low melting point (−108.9 °C) and boiling point (−4.4 °C) and exist as gas. Therefore, commercially available 1,3-butadiene standard solution (2 mg/mL in methanol) was used as the standard stock solution. Portions (1 mL) of 1,3-butadiene, isoprene, acrylonitrile stock solutions and portions (0.4 mL) of benzene and toluene stock solution were added into the 10 mL volumetric flask. After addition of 2 mL of carbon disulfide, the solution was diluted to 10 mL with methanol. Solvent of this standard mixture is the same as sample eluate and consists of 20% carbon disulfide and 80% methanol. This standard mixture solution contains 1,3-butadiene (0.2 mg/mL), isoprene (0.2 mg/mL), acrylonitrile (0.2 mg/mL), benzene (0.08 mg/mL) and toluene (0.08 mg/mL). After placing 0, 0.2, 0.4, 0.6, 0.8, 1.0 1 mL of standard mix solution in autosampler vials and diluting with 20% carbon disulfide methanol solution, 8 μL of benzene-*d*6 (10 mg/mL; internal standard) is added and analyzed by GC/MS under the conditions described in Section 2.1. Fig. 6 shows the total ion chromatograms of a mixed standard solution. All target compounds are completely separated under the analytical conditions of this study. Benzene-*d*6 (Internal standard) and benzene are partially overlapped but can be separated with mass chromatograms of *m*/*z* 84 and 78 fragments.

Preparation of standard solution for carbonyls was according to Health Canada method T-104 [31].

3.5. Limit of detection, limit of quantitation, reproducibility and recovery

The limit of detection (LOD) and limit of quantitation (LOQ) of a CX-572 cartridge method was calculated using linear regression theory [43]. 10 μL of VOC mixture standard solution (0.05 mg/mL each) and 10 μL of carbonyl mixture standard solution (0.5 mg/mL each) were introduced into the 10 CX-572 cartridges and analyzed using the analytical conditions described above. The LOD and LOQ were calculated as being three times the standard deviation obtained from the data of 10 samples (Table 1). The reproducibility of CX-572 cartridge method was estimated from data of 10 cartridges spiked with 10 μL of VOC mixture standard solution (5 mg/mL) and carbonyl mixture standard solution (50 mg/mL).

Table 1
LOD, LOQ, reproducibility and recovery of CX-572 cartridge method.

Compound	LOD (μg)	LOQ (μg)	RSD (%)	Recovery (%)
1,3-Butadiene	0.07	0.24	5.9	106
Isoprene	0.04	0.15	3.7	106
Acrylonitrile	0.07	0.22	5.6	98.1
Benzene	0.03	0.10	2.4	103
Toluene	0.03	0.09	2.1	99.3
Acetaldehyde	0.26	0.88	1.2	99.2
Acetone	0.15	0.49	1.5	103
Acrolein	0.13	0.45	2.2	102
Propanal	0.24	0.80	3.3	96.3
Crotonaldehyae	0.07	0.25	1.3	102
2-Butanone	0.14	0.46	1.7	102
Butanal	0.29	0.97	2.3	99.0
i-Valeraldehyde	0.31	1.1	3.2	95.9

The relative standard deviation (RSD) and recovery are shown in Table 1.

3.6. Measurement of real sample cigarettes

VOCs and carbonyls in mainstream cigarette smoke from reference cigarettes (CM6, 3R4F, 1R5F) were analyzed by CX-572 method. Table 2 shows the amounts of VOCs and carbonyls in mainstream cigarette smoke measured by CX-572 method. For reference, CORESTA data published at March 2013 [30,32] are also shown in parenthesis in Table 1. Formaldehyde was removed from the target compounds because it was trapped in the Cambridge filter pad before the CX-572 cartridge. We have confirmed that 63% of formaldehyde in mainstream cigarette smoke was trapped into the Cambridge filter pad. The Cambridge filter pad collects the particle fraction containing water. Formaldehyde is highly hydrophilic

Table 2
VOCs and carbonyl in mainstream cigarette smoke measured by CX-572 method. μg/cigarette. Data indicates the mean value ($n = 5$) of the CX-572 method and the parenthesis indicates the mean value from CORESTA [30,32].

Compound	CM6		3R4F		1R5F	
	ISO	HCI	ISO	HCI	ISO	HCI
Puff number	8.6	12	8.0	9.8	6.5	6.4
1,3-Butadiene	65 (61)	110 (110)	37 (40)	94 (100)	12 (12)	88 (91)
Isoprene	590 (560)	940 (1000)	310 (350)	750 (910)	110 (120)	750 (890)
Acrylonitrile	12 (12)	24 (25)	8.2 (8.5)	27 (27)	2.0 (2.1)	25 (28)
Benzene	70 (60)	120 (110)	47 (42)	110 (97)	15 (14)	88 (79)
Toluene	120 (87)	220 (170)	84 (67)	220 (170)	21 (19)	160 (140)
Acetaldehyde	670 (690)	1100 (1300)	570 (550)	1400 (1600)	200 (140)	1200 (1400)
Acetone	320 (270)	580 (520)	250 (210)	600 (600)	87 (63)	540 (490)
Acrolein	72 (69)	120 (130)	56 (48)	140 (160)	15 (9.3)	110 (120)
Propanal	62 (53)	120 (100)	49 (42)	130 (120)	16 (12)	100 (99)
Crotonaldehyde	25 (21)	53 (48)	15 (11)	51 (50)	2.0 (2.4)	42 (36)
2-Butanone	130 (62)	240 (130)	97 (52)	220 (150)	34 (14)	190 (110)
Butanal	37 (37)	68 (70)	26 (25)	65 (72)	10 (7.7)	57 (59)
i-Valeraldehyde	22 (n.a.)	49 (n.a.)	16 (n.a.)	48 (n.a.)	5.1 (n.a.)	40 (n.a.)

n.a.: not available.

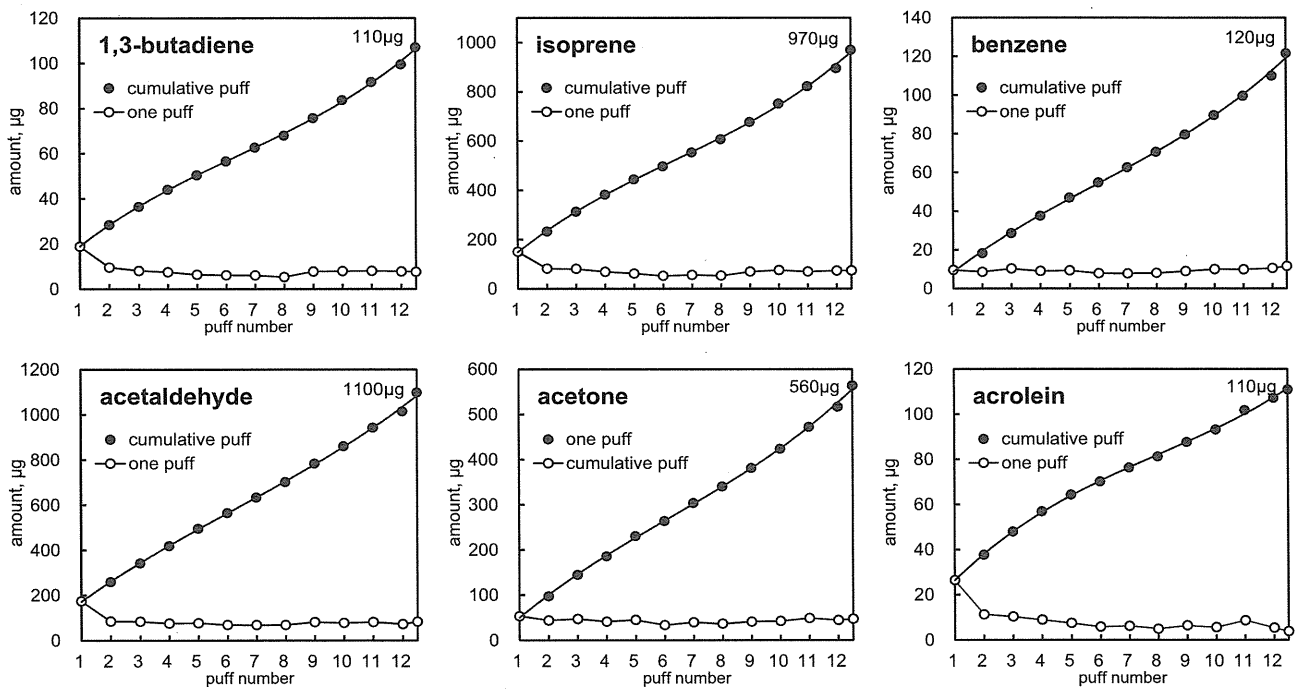

Fig. 7. Changes in amounts of VOCs and carbonyls in mainstream cigarette smoke with puff number. Open circles indicate every one puff data and closed circles indicate cumulative data.

and easy to solve into the Cambridge filter containing tar with water. It was suggested that DNPH-silica cartridge method without Cambridge filter pad is more suitable for collection of formaldehyde in mainstream cigarette smoke. All target compounds except for formaldehyde passed through the Cambridge filter pad.

Almost all measurement values by CX-572 method are very close to CORESTA data [30,32] except for 2-butanone. Measurement values of 2-butanone by CX-572 method are twofold higher than CORESTA data. In acidic aqueous solutions, 2-butanone-DNPhydrazone is hydrolyzed back to the 2-butanone and DNPH with water [44]. Impinger methods use DNPH solution containing 50% water (28 mol/L) for impinger and 2-butanone-DNPhydrazone may be hydrolyzed with water. In the CX-572 method, the hydrolysis of 2-butanone may be prevented because the eluate solution contains no water.

CX-572 method can measure VOCs and carbonyls in mainstream cigarette smoke from not only one whole cigarette but also from one puff volume because of its high sensitivity and simple operation. CX-572 cartridge installed in the smoking machine can be exchanged with new cartridge at 5 s within puff interval of HCI and ISO regime (30 s, 60 s). Fig. 7 shows the changes in amounts of VOCs and carbonyls in mainstream cigarette smoke with puff number while one cigarette had been smoked according to the HCI regime. Sample cigarette is CM6. Amounts of 1,3-butadiene, isoprene, acetaldehyde and acrolein at the first puff were twofold higher than second puff and after that, however, benzene, toluene, acetone and 2-butanone were not so varied at every puff. Cumulative data show cubic curve in Fig. 7 and the data of last puff correlates with the data from one cigarette. Cumulative values at last puff in Fig. 7 are very close to the one cigarette data described in Table 1.

4. Conclusions

A sorbent cartridge method (CX-572 method) for measurement of VOCs and carbonyls in cigarette mainstream cigarette smoke developed in this study has the advantages of high efficiency, high sensitivity, and very simple operation. CX-572 cartridge was shown to be capable of collection of VOCs and carbonyls in cigarette smoke at room temperature. Moreover, it can measure cigarette smoke not only from one whole cigarette but also from one puff volume because of its high sensitivity and simple operation.

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Evaluation of Genetic Polymorphisms in Patients with Multiple Chemical Sensitivity

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Abstract

Objective: Multiple chemical sensitivity (MCS) is a chronic medical condition characterized by symptoms that the affect an individual's response to low-level chemical exposure. In this study, we identified a chemical sensitive population (CSP) and investigated the effect of genetic polymorphisms on their risk of chemical sensitivity.

Methods: A quick environment exposure sensitivity (QEESI) questionnaire was used to survey 324 Japanese male workers whose DNA samples had been collected and stored. The following genes, which encode enzymes affecting the metabolic activation of a large number of xenobiotic compounds, were selected and analyzed in order to determine their influence on genetic predisposition to CSP: cytochrome P450 (CYP) 2E1, N-acetyl transferase (NAT) 2, glutathione S-transferase (GST) M1, GSTT1, GSTP1, low Km aldehyde dehydrogenase (ALDH2), and superoxide dismutase (SOD) 2.

Results: Significant case-control distributed differences were observed in *SOD2* polymorphisms and allele frequency distribution in high chemical sensitive subjects. Both the significant adjusted OR of 4.30 (95% CI, 1.23–15.03) and 4.53 (95% CI, 1.52-13.51) were observed in *SOD2* Ala/Ala and Val/Ala compared to Val/Val and in *SOD2* Ala/Ala compared to Val/Ala compared to Val/Val genetic analysis in the high chemical sensitivity case-control study.

Conclusions: We observed that high chemical sensitive individuals diagnosed by using Japanese criteria as MCS patients were more significantly associated with *SOD2* polymorphisms.

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Introduction

Individuals experiencing multiple chemical sensitivity (MCS) often report symptoms from various organs related to inhalation of multiple unrelated airborne chemicals in concentrations below what is normally associated with toxicological responses [1]. MCS is a chronic medical condition characterized by symptoms that affect an individual's responses to low-level chemical exposure. These chemicals can include pesticides, plastics, and paint fumes. Symptoms are usually vague and nonspecific, such as fatigue and headaches. In general the reported symptoms are attributed to previous chemical exposure and recur on subsequent exposure to similar or structurally unrelated chemicals at levels normally considered to be nontoxic [2]. The clinical characteristics of MCS patients are usually evaluated using questionnaires such as the environmental exposure and sensitivity inventory (EESI) questionnaire or clinical interviews that rely on the subject's retrospective self-reports [3]. Miller and Prihoda developed a globally standardized self-administered questionnaire, the quick environment exposure sensitivity (QEESI), which is designed to assist researchers and clinicians in screening, studying, and evaluating patients with MCS [4].

The variation in individual responses to multiple environmental chemicals is exceptionally wide. This variation is accounted for by differences in metabolic capacity, DNA repair capacity, or genetic predisposition. The existence of several well-known genetic polymorphisms affecting the activity of enzymes metabolizing xenobiotics have prompted research into whether these polymorphisms are associated with MCS and chemical sensitivity in general populations [5–8]. Either negative or significant association in genetic polymorphisms with MCS or self-reported chemical-related sensitivity was found in those studies.

Chemicals that enter cells are subjected to biotransformation by oxidative phase I enzymes in the cytoplasm as cytochrome P450 (CYP) s. CYP2E1 represents a major CYP isoform in the human liver and is also expressed in extrahepatic tissue. It can be induced by certain chemicals such as ethanol although large interindividual variations have been observed in its induction, suggesting that genetic polymorphisms may be involved [9]. A polymorphism in *Rsa*I, which is located in the 5′-flanking region of *CYP2E1* gene, was reported to be associated with the transcriptional regulation of gene expression [10]. The N-acetyl transferase (NAT) 2 enzyme plays an important role in the metabolism of aromatic and heterocyclic amines via *N*-acetylation and *O*-acetylation pathways, which are responsible for their activation and/or deactivation, respectively [11]. Functional variation of NAT2 leads to a slow or rapid acetylator phenotype. A study by Vineis et al. indicated that clearance of low-dose environmental carcinogens decreases the slow-acetylator phenotype of NATs [12]. Glutathione S-transferase (GST) s are multifunctional enzymes and the variant allele changes of these genes result in either total absence or a substantial change in enzyme activity [13]. The absence of GSTM1 and GSTT1 phenotypic activity is caused by homozygosity for an inherited deletion of these genes, referred to as the null genotype [14]. The Ile105Val (A to G substitution replacing isoleucine with valine polymorphism) in the *GSTP1* gene has been found to modify the enzyme's specific activity and affinity for electrophilic substrates [15]. Low Km aldehyde dehydrogenase (ALDH2) predominantly located in mitochondria is characterized by being responsible for the oxidation of most of the acetaldehyde generated during alcohol metabolism [16]. Approximately half of the Japanese population lacks ALDH2 activity, and the enzyme deficit of ALDH2 causes a significant change in the rate of ethanol metabolism [17]. This is often caused by the ALDH *2($ALDH2_{K504}$) mutant allele. Individuals possessing either 1 or 2 copies of the mutant allele show alcohol-related sensitivity responses, including facial flushing and hangovers [18].

Many lifestyles are closely associated with oxidative stress, which is augmented by smoking, drinking, and an irregular diet. Many environmental factors can also generate oxygen radicals that induce DNA damage and reactive oxygen species (ROS) production [19]. Superoxide dismutase (SOD) enzymes act as antioxidants and protect cellular components from being oxidized by reactive oxygen species (ROS) [20]. SOD plays a pivotal role in protecting cells from free radicals and oxidative damage. SOD2 is one of the major superoxide scavengers in mitochondria, where it catalyzes accumulated superoxide radicals into H_20_2 [21]. The 47 cytosine-to-thymine (47C→T) transition at codon 16 in the *SOD2* gene creates a sense mutation of alanine-to-valinein the SOD2 protein [22].

Materials and Methods

Study Characteristics

The present study was conducted at a paper pulp producing company between 2002 and 2006 in Kyushu, Japan. A total of 324 male subjects whose purified DNA had been obtained and stored by the company were asked to complete QEESI questionnaires and were eligible for this study. Subjects with a diagnostic history of cancer, chronic obstructive lung disease, cardiovascular disorder or diabetes were excluded from the study. All study subjects completed the QEESI questionnaire, which also covered the history of drinking and smoking. The distribution of smoking, drinking, age, diagnosis history, score status of survey subscales, and the CSP-diagnosed criterion of the participants are presented in Table 1.

Table 1. Characteristics of the entire study population and the distribution of QEESI subscales.

		n	%
Age	<40	83	25.6%
	40–49	95	29.3%
	≥50	146	45.1%
Average age (years ± SD[a])	46.84 ± 8.79		
Smoking	Non-smoker	137	42.3%
	Smoker	187	57.7%
Drinking	Non-drinker	62	19.1%
	Drinker	262	80.9%
History of diagnosis	Multiple chemical sensitivity (MCS)	3	0.9%
	Allergy	61	18.8%
	Sick house	0	0.0%
	Non	260	80.3%
QEESI[b] subscales			
Chemical sensitivity	0 score	113	34.9%
	1–39 score	182	56.2%
	≥40 score (cut-off value)	29	8.9%
Symptom severity	0 score	53	16.4%
	1–19 score	182	56.1%
	≥20 score (cut-off value)	89	27.5%
Life impact	0 score	176	54.3%
	1–9 score	90	27.8%
	≥10 score (cut-off value)	58	17.9%
CSP[c] cases	All	116	100%
Low chemical sensitivity	Cut-off value subscale = 1	67	57.8%
Middle chemical sensitivity	Cut-off value subscale = 2	38	32.7%
High chemical sensitivity	Cut-off value subscale = 3	11	9.5%
Controls	Cut-off value subscale = 0	208	64.2%

[a]. Standard deviation

[b]. Quick Environment Exposure Sensitivity

[c]. Chemical sensitive population

Survey instruments

We used the QEESI questionnaire (Japanese version) translated by Ishikawa and Miyata for the survey [23]. We used this diagnostic instrument to define the chemical sensitive population (CSP) in this study and divided them into 3 case groups according to the scores achieved by cut-off values of the QEESI subscales. Each criterion subscale of QEESI has 10 questions, and each question has a possible score of 0–10. Therefore, the total possible score of each subscale was 0–100.

The chemical sensitivity section of the survey asked the subjects to list various odors or chemical exposures that made them feel sick (items like diesel or gas engine exhaust; gasoline; or insecticide). The symptom severity section asked about symptoms the subjects may have experienced commonly (items like problems with muscles or joints, such as pain, aching, cramping, stiffness, or weakness; problems with your head, such as headaches or a feeling of pressure or fullness in your face or head; problems with your skin, such as a rash, hives, or dry skin). The life impact section of the survey asked if the subjects were sensitive to certain chemicals or foods and if the sensitivities had affected various aspects of their life (items like your diet; your ability to work or go to school; your choice of clothing).

Hojo et al. designed a study to establish the cut-off value for Japanese using the QEESI for screening MCS patients [24]. The cut-off values for Japanese subjects were determined to be ≥40 for the chemical sensitivity subscale, ≥20 for the symptom severity subscale, and ≥10 for the life impact subscale. By using these 3 criteria, we divided our subjects into 3 case groups and 1 control group. Individuals who were 1 subscale from the cut-off value were defined as low chemical sensitivity; those who were 2 subscales from the cut-off value were defined as middle chemical sensitivity; those who were 3 subscales from the cut-off value were defined as high chemical sensitivity; those who were 0 subscales from the cut-off value were defined as the controls. Scores for each subscale in the controls were as follows: chemical sensitivity <40, symptom severity <20 and life impact <10. Therefore, they were not compliant with any of the diagnostic criteria in QEESI, and were not diagnosed with MCS before enrolment in this study. In addition to QEESI, the diagnosis history of MCS, sick house syndrome, allergic disease, and age were also included in the study survey.

Genotyping

DNA isolation. Genomic DNA was isolated from whole blood using the Viogene® Blood and Tissue Genomic DNA Extraction Miniprep system (Viogene, Japan) according to the manufacturer's instructions and stored at −20° C.

Genotyping assay. Genes encoding enzymes affecting the biotransformation of a large number of xenobiotic compounds were selected for determination of their ability to predispose to CSP, namely, CYP2E1, NAT2, GSTM1, GSTT1, GSTP1, ALDH2, and SOD2.

The *CYP2E1* genetic polymorphism was determined by polymerase chain reaction (PCR) amplification run on a GeneAmp PCR System 9700 (Applied Biosystems), followed by digestion with *Rsa*I (Takara Bio, Japan) since the predominant allele (C1) is sensitive while the C2 allele is resistant to *Rsa*I digestion [9]. The *CTP2E1* genotypes were categorized as the homozygous genotype of C1/C1 or C2/C2 and the heterozygous genotypes of C1/C2. The *NAT2* alleles examined were WT (*4, wild-type), M1 (*5), M2 (*6), and M3 (*7). The *5, *6, and *7 single nucleotide variations have all been associated with decreased enzyme activity [25]. The NAT2 single nucleotide polymorphisms (SNPs) of *6 (rs1799930, Applied Biosystems assay ID:C__1204091_10) and *7 (rs1799931, Applied Biosystems assay ID:C__572770_20) alleles were analyzed by real-time PCR. The *5 allele was analyzed by PCR restriction fragment length polymorphism (PCR-RFLP) and digestion with the restriction enzyme *Kpn*I (Takara Bio, Japan). The NAT2 rapid acetylator genotypes are those with none mutant alleles (*4/ *4); slow acetylator genotypes are those with 2 mutant alleles (*5/ *5, *5/ *6, *5/ *7, *6/ *6, *6/ *7, and *7/ *7); intermediate acetylator genotypes are those with 1 mutant allele (*4/ *5, *4/ *6, and *4/ *7). A multiplex PCR method was used to detect the presence or absence of GSTM1 and GSTT1. In this method, both the GST and β-globin primers (Sigma-Aldrich, Japan) are included in the same PCR reaction. The absence of a 219-bp band indicates the *GSTM1* null genotype, and the absence of a 480-bp band indicates the *GSTT1* null genotype; β-globin was coamplified in all samples [14]. The *GSTP1* genotype was determined by using PCR-RFLP as previously described [26]. The *GSTP1* genotypes were categorized as the homozygous genotypes of A/A or G/G and the heterozygous genotype of A/G. The *ALDH2* E504K polymorphism (SNP: rs671, Applied Biosystems assay ID:C__11703892_10), and the *SOD2* Val16Ala polymorphism (SNP: rs4880, Applied Biosystems assay ID:C__8709053_10) were analyzed by real-time PCR. All real-time PCR reactions were performed in 48-well plates on a StepOne Real-Time PCR system (Applied Biosystems) and were prepared by using a TaqMan® Universal PCR Master Mix, TaqMan® SNP genotyping assay mix, DNase-free water, and 10 ng of genomic DNA in a final volume of 10 μL per well. The cycling conditions were 30 s at 60° C, 10 min at 95° C, 35 cycles of 15 s at 95° C and 1 min at 60° C, with a final step of 30 s at 60° C. The standard mode reaction time was 90 min. The allelic discrimination results were determined after the amplification by performing an end-point read in an allelic discrimination graph (VIC on abscissa, FAM on ordinate). The genotypes of *ALDH2* were categorized as the homozygous genotypes of *1/ *1 or *2/ *2 and the heterozygous genotype of *1/ *2. The genotypes of *SOD2* were categorized as the homozygous genotypes of Val/Val or Ala/Ala and the heterozygous genotype of Val/Ala.

Statistical analysis

Statistical power calculations were performed using Epistat (Finnish Institute of Occupational Health). The study sample size had at least 80% power (two-sided test significant, α of 0.05) to detect an OR of at least 2.5, following the calculations used in previous studies [9,14,16,25–27]. The distribution of each genetic variant in the cases and controls was first assessed for consistency with the Hardy-Weinberg equilibrium (HWE) on a contingency table of observed-versus-predicted genotype frequencies by using Chi-Square Goodness of Fit test.

The frequency distribution of genotypes and alleles in cases and controls was determined by analysis using the Pearson's chi-square test or Fisher's exact test. To determine an association between each SNP and CSPs, we computed the overall genotypic test of association in the dominant or recessive genetic models and in the additive genetic models. Relative association in the case-control designs were assessed

by calculating odds ratios (ORs) and 95% confidence intervals (95% CIs) in logistic regression analyses; two-tailed *P* values <0.05 were considered statistically significant. Statistical analysis was carried out using SPSS version 18 (SPSS, Japan).

Ethical Statement

The Ethics Review Boards of Miyazaki University (no. 82; April 9, 2003) and Kumamoto University (no. 168; May 11, 2011) approved this study, following the ethical guidelines for human genome research. All participants provided their written informed consent to participate in this study, and the complete protection of their personal data was agreed in a written form.

Results

Table 1 presents the diagnostic history frequencies of MCS, sick house syndrome, and allergic diseases of the study participants. Three subjects reported a history of diagnosis for MCS. None of the subjects in this study population had been diagnosed with sick house syndrome. The percentage of subjects that had QEESI cut-off values applying to the subscales of chemical sensitivity, symptom severity, and life impact were 8.9%, 27.5%, and 17.9% respectively. In CSP cases, 57.8% of the subjects were defined as low chemical sensitivity; 32.7%, as middle chemical sensitivity; and 9.5%, as high chemical sensitivity. Individuals previously diagnosed with MCS were all defined as the CSP case group, one of them was classified as high chemical sensitivity.

The genotype distributions of *CYP2E1*, *NAT2*, *GSTP1*, *ALDH2*, and *SOD2* in all the cases and controls were checked and found to not differ significantly from those predicted by the Hardy–Weinberg law ($p > 0.05$), indicating no evidence of non-random selection. The Hardy-Weinberg equilibrium could not be tested in *GSTM1* and *GSTT1* polymorphisms because of the inability of the present PCR protocol to separate heterozygous carriers of the deletion polymorphisms. There was no significant difference in the distribution of drinking and smoking statuses between cases and controls (Table 2). The proportion of low chemical sensitive individuals (Case 1) who were over 50 years old was significantly greater in the cases than in the controls (56.7% vs. 39.9%). There was no significant difference in the mean age between Case 2 group and control group, also no significant difference was found between Case 3 group and control group ($p > 0.05$, student's t test, data not shown). Significant frequency differences in *SOD2* genotypes and alleles were observed in high chemical sensitive individuals compared to controls. Instead of the genotype, only the allele-frequency difference in *GSTP1* was observed in middle chemical sensitive individuals compared to controls.

In addition, we determined the association between genetic polymorphisms and CSP risk in the 3 case-control designs using logistic regression analyses as shown in Table 3. Both crude ORs and adjusted ORs (adjusted by age, smoking, and drinking) were calculated. In the high chemical sensitivity (Case 3) case-control study, the significant crude OR of 3.90 (95% CI, 1.14–13.31, $p = 0.03$) and the adjusted OR of 4.30 (95% CI, 1.23–15.03, $p = 0.02$) was observed in *SOD2* Ala/Ala and Val/Ala variants compared to the Val/Val genotype. The significant crude OR of 3.67 (95% CI, 1.32-10.20, $p = 0.01$) and adjusted OR of 4.53 (95% CI, 1.52-13.51, $p = 0.01$) were observed in *SOD2* additive genetic model analysis of Ala/Ala vs. Val/Ala vs. Val/Val in the Case 3 study. The *GSTP1* genetic analysis of G/G vs. A/G vs. A/A obtained a significant crude OR in the Case 2 study. However, the OR value decreased and lost statistical significance after adjusting by age, smoking, and drinking status.

Discussion

This study focused on determining if there were any associations between chemical sensitivity and genetic polymorphisms. There were no significant associations between chemical sensitivity and the genetic polymorphisms in the previously studied genes—*NAT2, GSTM1, GSTT1*—although there was a significant association between middle chemical sensitivity and the genetic polymorphism in *GSTP1* before adjusting for other confounding factors. In addition, no significant results were obtained when the genetic polymorphisms in *CYP2E1* and *ALDH2* were compared to chemical sensitivity. However, results from this case-control study indicated that there was an increased risk of high chemical sensitivity associated with *SOD2* Ala allele genotypes. The diagnostic criterion for high chemical sensitivity fit with all the 3 cut-off values of the QEESI subscales that are used to diagnose MCS patients using Japanese criteria [24].

CYP2E1 is a major contributor to ethanol-induced oxidant stress and to ethanol-induced liver injury [28]. Individuals with the C2/C2 genotype have higher expression of CYP2E1 mRNA than C1/C1 genotype subjects [29]. Although we predicted that the genetic variant of CYP2E1 might be associated with ethanol-induced CSP, no significant association was found. This may be due to the fact that even though the participants in this study were slightly exposed to ethanol-related xenobiotic chemicals such as alcohol, it did not lead to excessive oxidant stress. The *NAT2* genetic polymorphisms associated with chemical sensitivity have been investigated in several studies with inconsistent results. The effect of NAT2 activity was not found in the case-control study of MCS by Berg et al [5]. However, the fast NAT2 acetylator was associated with self-reported chemical sensitivity only in the most severely affected group (OR = 3.1, $p = 0.04$) [5]. The study by McKeown-Eyssen et al. also showed that NAT2 rapid acetylator (OR = 4.14; $p = 0.01$) was significantly higher in MCS cases than in controls [8]. The *NAT2* slow or inter compared to rapid acetylator genotype showed gradually lower OR from low to high chemical sensitivity cases in this study, but no statistical significance was found. This may be due to the different characteristics of the research subjects; the genetic polymorphisms of *NAT2* may not have been sensitive enough in our case-control designs. *GST* genetic polymorphisms have been associated with atopy (allergy, asthma, and atopic dermatitis) [13]. A study by Mapp et al. showed that the *GSTP1* genetic polymorphism is associated with asthma and airway hyperresponsiveness [30]. Schnakenberg et al. suggested that *GSTM1* and *GSTT1* genes

Table 2. Distribution of age, smoking, drinking, and genotypes in chemical sensitive population (CSP) cases and controls.

		Controls (%)	Case 1 (%)	P[a,b]	Case 2 (%)	P[a,b]	Case 3 (%)	P[a,c]
			Low chemical sensitivity		Middle chemical sensitivity		High chemical sensitivity	
		n = 208	n = 67		n = 38		n = 11	
Age	<40	61 (29.3%)	9 (13.4%)		9 (23.7%)		4 (36.4%)	
	40–49	64 (30.8%)	20 (29.9%)		9 (23.7%)		2 (18.2%)	
	≥50	83 (39.9%)	38 (56.7%)	0.02	20 (52.6%)	0.34	5 (45.4%)	0.74
Smoking	Non-smoker	83 (39.9%)	31 (46.3%)		16 (42.1%)		7 (63.6%)	
	Smoker	125 (60.1%)	36 (53.7%)	0.36	22 (57.9%)	0.80	4 (36.4%)	0.11
Drinking	Non-drinker	38 (18.3%)	12 (17.9%)		10 (26.3%)		2 (18.2%)	
	Drinker	170 (81.7%)	55 (82.1%)	0.95	28 (73.7%)	0.25	9 (81.8%)	0.68
NAT2								
Genotype	Rapid	90 (43.3%)	36 (53.7%)		20 (52.6%)		7 (63.6%)	
	Inter + Slow	118 (56.7%)	31 (46.3%)	0.14	18 (47.4%)	0.29	4 (36.4%)	0.16
GSTM1								
Genotype	non-null	121 (58.2%)	37 (55.2%)		18 (47.4%)		4 (36.4%)	
	homozygous-null	87 (41.8%)	30 (44.8%)	0.67	20 (52.6%)	0.22	7 (63.6%)	0.13
GSTT1								
Genotype	non-null	84 (40.4%)	31 (46.3%)		19 (50.0%)		5 (45.5%)	
	homozygous-null	124 (59.6%)	36 (53.7%)	0.40	19 (50.0%)	0.27	6 (54.5%)	0.49
GSTP1								
Genotype	A/A	154 (74.0%)	48 (71.6%)		23 (60.5%)		6 (54.5%)	
	G/G + A/G	54 (26.0%)	19 (28.4%)	0.70	15 (39.5%)	0.09	5 (45.5%)	0.14
Allele	A	358 (86.1%)	114 (85.1%)		58 (76.3%)		17 (77.3%)	
	G	58 (13.9%)	20 (14.9%)	0.78	18 (23.7%)	0.03	5 (22.7%)	0.20
CYP2E1								
Genotype	C1/C1	117 (56.2%)	39 (58.2%)		27 (71.1%)		8 (72.7%)	
	C2/C2 + C1/C2	91 (43.8%)	28 (41.8%)	0.78	11 (28.9%)	0.09	3 (27.3%)	0.23
Allele	C1	307 (73.8%)	102 (76.1%)		62 (81.6%)		19 (86.4%)	
	C2	109 (26.2%)	32 (23.9%)	0.59	14 (18.4%)	0.15	3 (13.6%)	0.19
ALDH2								
Genotype	*1/*1	125 (60.1%)	39 (58.2%)		25 (65.8%)		9 (81.8%)	
	*2/*2 + *1/*2	83 (39.9%)	28 (41.8%)	0.78	13 (34.2%)	0.51	2 (18.2%)	0.13
Allele	*1	326 (78.4%)	105 (78.4%)		60 (78.9%)		20 (90.9%)	
	*2	90 (21.6%)	29 (21.6%)	1.00	16 (21.1%)	0.91	2 (9.1%)	0.12
SOD2								
Genotype	Val/Val	159 (76.4%)	52 (77.6%)		28 (73.7%)		5 (45.5%)	
	Ala/Ala + Val/Ala	49 (23.6%)	15 (22.4%)	0.84	10 (26.3%)	0.71	6 (54.5%)	0.03
Allele	Val	365 (87.7%)	116 (86.6%)		65 (85.5%)		15 (68.2%)	
	Ala	51 (12.3%)	18 (13.4%)	0.72	11 (14.5%)	0.59	7 (31.8%)	0.02

[a]. *p* value <0.05 is considered statistically significant

[b]. Pearson's chi-square test

[c]. Fisher's exact test

were significantly deleted homozygously more often (homozygous-null) in self-reported chemical sensitivity cases than in controls, although no case-control differences were observed in the genotype frequencies of *GSTP1* [7]. No significant differences between the genetic polymorphisms of *GSTT1* and *GSTM1* were observed in the present study, but a gradually higher OR was found in *GSTM1* homozygous-null compared to non-null genotype from low to high chemical sensitivity cases. In the case 2 study, significant results were observed in the *GSTP1* allele frequency and in the crude OR, although the logistic regression analysis lost significance after adjusting for confounding factors. In a review study, cleaning agents, pesticides, perfumes, and vehicle exhaust were the products most often reported to trigger MCS that were not due to smoking and drinking [31]. The triggers of symptoms of middle chemical sensitivity cases defined by this study may not be those from the most often reported MCS trigger but were also influenced by multiple factors such as age, smoking and drinking which may be predominantly associated with the *GSTP1* genetic polymorphism. Further research and larger sample sizes will be necessary to evaluate the statistical significance of the interaction between the *GSTP1* genetic polymorphisms and CSPs by stratification according to these multiple factors. Alcohol consumption is associated with many

Table 3. Odds ratios of chemical sensitive population (CSP) cases compared to controls categorized by genotype.

Variable[a]	Control	Case 1 (Low chemical sensitivity)				Case 2 (Middle chemical sensitivity)				Case 3 (High chemical sensitivity)			
	n=208	n=67				n=38				n=11			
		OR (95% CI)	P[c]	OR[b] (95% CI)	P[c]	OR (95% CI)	P[c]	OR[b] (95% CI)	P[c]	OR (95% CI)	P[c]	OR[b] (95% CI)	P[c]
		Crude		Adjusted		Crude		Adjusted		Crude		Adjusted	
NAT2													
Genotype Slow + Inter vs. Rapid	1[d]	0.66 (0.38–1.14)	0.14	0.68 (0.38–1.19)	0.17	0.69 (0.34–1.37)	0.29	0.68 (0.34–1.36)	0.27	0.44 (0.12–1.54)	0.20	0.48 (0.13–1.70)	0.25
Slow vs. Inter vs. Rapid	1[d]	0.70 (0.43–1.12)	0.13	0.70 (0.43–1.14)	0.15	0.68 (0.37–1.24)	0.21	0.66 (0.36–1.22)	0.18	0.43 (0.13–1.38)	0.16	0.47 (0.14–1.51)	0.20
GSTM1													
Genotype homozygous-null vs. non-null	1[d]	1.13 (0.65–1.96)	0.67	1.09 (0.62–1.93)	0.76	1.55 (0.77–3.09)	0.22	1.51 (0.75–3.04)	0.25	2.43 (0.69–8.57)	0.17	2.34 (0.66–8.30)	0.19
GSTT1													
Genotype homozygous-null vs. non-null	1[d]	0.79 (0.45–1.37)	0.40	0.75 (0.42–1.32)	0.32	0.68 (0.34–1.36)	0.27	0.66 (0.33–1.33)	0.24	0.81 (0.24–2.75)	0.81	0.84 (0.25–2.87)	0.78
GSTP1													
Genotype G/G + A/G vs. A/A	1[d]	1.13 (0.61–2.09)	0.70	1.06 (0.57–1.98)	0.86	1.86 (0.91–3.82)	0.09	1.76 (0.85–3.64)	0.13	2.38 (0.70–8.10)	0.17	2.48 (0.72–8.55)	0.15
G/G vs. A/G vs. A/A	1[d]	1.08 (0.62–1.89)	0.78	1.02 (0.58–1.81)	0.93	1.88 (1.04–3.41)	0.04	1.79 (0.98–3.28)	0.06	1.85 (0.64–5.31)	0.26	1.95 (0.66–5.75)	0.23
CYP2E1													
Genotype C2/C2 + C1/C2 vs. C1/C1	1[d]	0.92 (0.53–1.61)	0.78	0.96 (0.54–1.71)	0.90	0.52 (0.25–1.11)	0.09	0.50 (0.24–1.08)	0.08	0.48 (0.12–1.87)	0.29	0.52 (0.13–2.06)	0.35
C2/C2 vs. C1/C2 vs. C1/C1	1[d]	0.89 (0.58–1.38)	0.61	0.94 (0.59–1.47)	0.78	0.67 (0.37–1.20)	0.18	0.65 (0.35–1.18)	0.16	0.47 (0.14–1.57)	0.22	0.50 (0.15–1.67)	0.26
ALDH2													
Genotype *2/*2 + *1/*2 vs. *1/*1	1[d]	1.08 (0.62–1.89)	0.78	1.02 (0.55–1.89)	0.95	0.78 (0.38–1.62)	0.51	0.61 (0.27–1.36)	0.22	0.34 (0.07–1.59)	0.17	0.26 (0.05–1.40)	0.12
*2/*2 vs. *1/*2 vs. *1/*1	1[d]	1.00 (0.61–1.65)	1.00	0.95 (0.54–1.67)	0.85	0.97 (0.53–1.77)	0.91	0.78 (0.40–1.53)	0.47	0.34 (0.08–1.53)	0.16	0.27 (0.05–1.35)	0.11
SOD2													
Genotype Ala/Ala + Val/Ala vs. Val/Val	1[d]	0.94 (0.49–1.81)	0.84	0.89 (0.46–1.75)	0.74	1.16 (0.53–2.55)	0.71	1.09 (0.49–2.42)	0.84	3.90 (1.14–13.31)	0.03	4.30 (1.23–15.03)	0.02
Ala/Ala vs. Val/Ala vs. Val/Val	1[d]	1.11 (0.63–1.96)	0.72	1.03 (0.58–1.85)	0.91	1.22 (0.60–2.50)	0.59	1.11 (0.54–2.30)	0.78	3.67 (1.32–10.20)	0.01	4.53 (1.52–13.51)	0.01

[a]. OR, odds ratio; 95% CI, 95% confidence interval

[b]. Odds Ratios were adjusted by age, smoking, and drinking

[c]. *p* value <0.05 is considered statistically significant

d. Reference category

health problems, including alcohol-related metabolic syndrome and hypertension [32]. The SNP rs671 in the ALDH2 gene showed the strongest association with drinking behavior in Japanese samples [33]. We failed to find a significant association between *ALDH2* or drinking status and CSP risk. This may be due to the fact that alcohol consumption was very common in the research participants, which were composed of mostly middle-aged men.

Overexpression of SOD2 is associated with increased levels of H_2O_2, a major contributor to oxidative stress [34]. In addition, H_2O_2 can also be transformed into hypochlorous acid (HOCl) through a myeloperoxidase (MPO)-catalyzed reaction, thus inducing cell damage [35]. The Ala-variant of *SOD2* allows more efficient SOD2 importation into the mitochondria. This generates more active SOD2 compared with the Val-variant and is related to the induction of oxidative stress [36,37]. We predicted that the *SOD2* genetic polymorphismis related to oxidative stress and associated with the CSP risk. In the high chemical sensitivity group, a significantly high crude OR of 3.67 was observed in the *SOD2* Ala allele compared to the Val allele carriers in the additive genetic analysis, and increased to 4.53 after adjusting for other confounding factors. This result may indicate that the Ala-variant genotypes are associated with elevated SOD2 activity together with increasing oxidative stress and increased chemical sensitivity risk.

Furthermore, in a clinical-based investigation, women were found to be more susceptible to MCS than men [5,38]. In contrast, a population-based investigation found no gender

differences for MCS [39]. The CSP research subjects in this study were composed entirely of men, unlike the study population in previous studies. We did not match age status to determine the age-distribution differences in the case control studies in reference to a previous study, which stated that MCS patients were found to be significantly older when compared to controls [5]. In our study, the proportion of individuals over 50 years old was greater in the low chemical sensitivity cases, but no significant age difference was found in the middle or high chemical sensitivity cases. In particular, we did not select the MCS patients diagnosed in hospital as cases in this study. We are currently investigating the chemical sensitivity status and the genetic polymorphisms related to chemical sensitivity in a normal working population.

Conclusion

The aim of this study was to examine the association between chemical sensitivity and genetic polymorphisms in *CYP2E1*, *NAT2*, *GSTM1*, *GSTT1*, *GSTP1*, *ALDH2*, and *SOD2*. In conclusion, we observed that the high chemical sensitive individuals who were also diagnosed by Japanese QEESI criteria as MCS patients were more significantly associated with *SOD2* polymorphisms. We hypothesize that our results reflect the gene-environment associations of increased chemical sensitivity in individuals, but further studies are needed to verify our observations. We were unable to confirm previous findings of substantial importance of genetic polymorphisms in *GSTM1*, *GSTT1* and *NAT2* to chemical sensitivity, but the research data are an important reference open to further exploration. A possible weakness of our study design is the lack of assessment of the environmental exposure to chemicals metabolized by the enzymes we studied. In addition, the participants of this study were limited to men from the same work site, and the cases were not classified according to any confounding factors due to sample size restriction. In future studies, we hope to classify research subjects into multiple categories according to confounding factors in order to examine the relationship between genetic polymorphisms and chemical sensitivity.

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Author Contributions

Conceived and designed the experiments: TK XC. Performed the experiments: XC XL MH MO WM. Analyzed the data: XC. Wrote the manuscript: XC.

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Notes

Determination of Carbonyl Compounds Generated from the E-cigarette Using Coupled Silica Cartridges Impregnated with Hydroquinone and 2,4-Dinitrophenylhydrazine, Followed by High-Performance Liquid Chromatography

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Carbonyl compounds in E-cigarette smoke mist were measured using coupled silica cartridges impregnated with hydroquinone and 2,4-dinitrophenylhydrazine, followed by high-performance liquid chromatography. A total of 363 E-cigarettes (13 brands) were examined. Four of the 13 E-cigarette brands did not generate any carbonyl compounds, while the other nine E-cigarette brands generated various carbonyl compounds. However, the carbonyl concentrations of the E-cigarette products did not show typical distributions, and the mean values were largely different from the median values. It was elucidated that E-cigarettes incidentally generate high concentrations of carbonyl compounds.

Keywords E-cigarette, carbonyl compounds, acrolein, glyoxal, methylglyoxal, glycerol, propylene glycol

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Introduction

E-cigarettes (electronic cigarettes or e-cigs) are battery-powered devices designed to deliver nicotine to a smoker in the form of a vapor, and were first introduced into the Chinese market in 2004. Currently, they are widely used around the world. In the United States, as of 2011, approximately 21% of adults who smoked traditional cigarettes had used electronic cigarettes, which was an increase from 10% in 2010, according to a study released by the Centers for Disease Control and Prevention.[1] Overall, approximately 6% of all adults have tried E-cigarettes, and these estimates are nearly double those from 2010.[1] It was reported in the news media in 2013 that electronic cigarettes were beginning to gain cultural acceptance, and sales were growing rapidly.[2]

An electronic cigarette contains three essential components: a plastic cartridge that serves as a mouthpiece and a reservoir for a liquid, an "atomizer" that vaporizes the liquid, and a battery. The liquid used to produce the vapor in electronic cigarettes is a solution of propylene glycol and/or glycerin and/or polyethylene glycol mixed with concentrated flavors and, optionally, a variable percentage of liquid nicotine concentrate. These base liquids have been widely used as food additives, as base solutions in personal care products, such as toothpaste, and in medical devices, such as asthma inhalers. However, there are few reports on chemical compounds in E-cigarette smoke mist; moreover, the health effects of inhaling nicotine vapor into the lungs are uncertain.

We have developed a new method (the HQ-DNPH method) for the determination of acrolein and other carbonyl compounds in cigarette smoke using coupled silica cartridges impregnated with hydroquinone and 2,4-dinitrophenylhydrazine[3] (DNPH), and we reported that E-cigarettes sometimes accidentally generate various carbonyl compounds, such as formaldehyde, acetaldehyde, acrolein, glyoxal, and methyl glyoxal.[3,4] In these previous studies, we concluded that ethylene glycol was oxidized to formaldehyde and glyoxal; propylene glycol was oxidized to formaldehyde, acetaldehyde, and methylglyoxal; and glycerol was oxidized to formaldehyde, acrolein, glyoxal, and methylglyoxal.[4] In this study, we determined the concentration of various carbonyl compounds generated from a total of 363 E-cigarettes (13 brands). The results are presented herein.

Experimental

Apparatus and reagents

An HPLC system (Shimadzu, Kyoto, Japan) with two LC-20AD pumps, an SIL-20AC autosampler and an SPD M20A photodiode array detector, was used. The analytical column was an Ascentis Express RP-Amide (2.7 μm particle size, 150 mm × 4.6 mm i.d., Supelco Inc., Bellefonte, PA). The column temperature was 40°C, and the injection volume was 10 μL. Solution A of the mobile phase mixture was composed of acetonitrile/water (40/60 v/v) containing 5 mmol/L ammonium acetate; solution B was composed of acetonitrile/water (75/25 v/v). HPLC elution was carried out with 100% A for 8 min, followed by a linear gradient from 100% A to 100% B in 37 min, and then maintained constant for 15 min using 100% B. The flow rate of the mobile phase was 0.7 mL/min.

An LM1/PLUS (Borgwaldt Technik GmbH, Hamburg,

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Germany) smoking machine was used for the collection of cigarette smoke.

The water used for the HPLC analysis and sample preparation was deionized and purified using a Milli-Q Water System equipped with a UV lamp (Millipore, Bedford, MA). 2,4-Dinitrophenylhydrazine hydrochloride (>98%) was obtained from Tokyo Kasei Co., Ltd. (Tokyo, Japan). Acetonitrile (HPLC grade, >99.9%), ethanol (>99.5%), hydroquinone (>99%), phosphoric acid (85% solution in water), and ammonium acetate (99.999%) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). The silica gel (spherical, 60/80 mesh, 120 Å mean pore size) was acquired from AGC Si-Tech. Co., Ltd. (Fukuoka, Japan).

The DNPH-impregnated silica cartridge (DNPH-cartridge) and the hydroquinone-impregnated silica cartridge (HQ-cartridge) were prepared according to previous reports.[3,4]

Collection and analysis of E-cigarette smoke

Before collecting smoke from the E-cigarettes, an HQ-cartridge and a DNPH-cartridge were connected. The coupled cartridges were then connected between the mouthpiece of the E-cigarette and the smoking machine, and the smoke from the E-cigarette was drawn into the coupled cartridges from the HQ-cartridge to the DNPH-cartridge according to the Canadian intense regimen;[5] (55 mL puff volume, 2-s puff duration, 30-s puff interval, and 10 puffs). After collection, the coupled cartridges were extracted using acetonitrile containing 1% phosphoric acid in a direction opposite to the air sampling direction until the total volume of the solution was 4.5 mL. After 10 min, ethanol (0.5 mL) was added to the eluate, and the solution was analyzed by HPLC. If the extraction was not performed immediately, the HQ-DNPH cartridge set was decoupled, and the individual cartridges were capped with stoppers.

Results and Discussion

Analysis of E-cigarette smoke by the HQ-DNPH method

Various types of carbonyl compounds were detected in the E-cigarette smoke. Figure 1 shows a representative chromatogram of a sample eluate by HPLC analysis with UV (360 nm) detection. In the HQ-DNPH method, it is possible to analyze C1 - C10 carbonyl compounds, and C1 - C3 carbonyl compounds, such as formaldehyde, acetaldehyde, acetone, acrolein, propanal, glyoxal, and methylglyoxal, were detected.

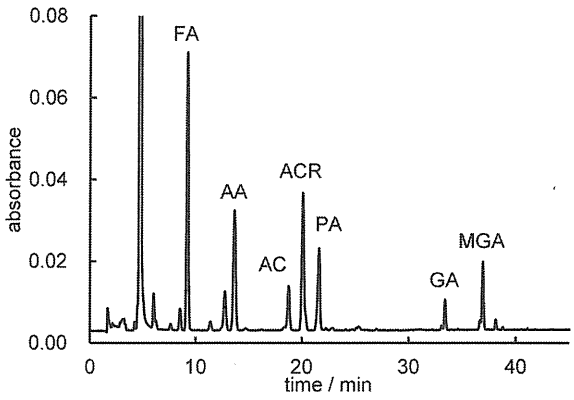

Fig. 1 Representative chromatogram of carbonyl DNPhydrazones derivatized from DNPH with carbonyls found in E-cigarette smoke. FA, formaldehyde; AA, acetaldehyde; AC, acetone; ACR, acrolein; PA, propanal; GA, glyoxal; MGA, methylglyoxal.

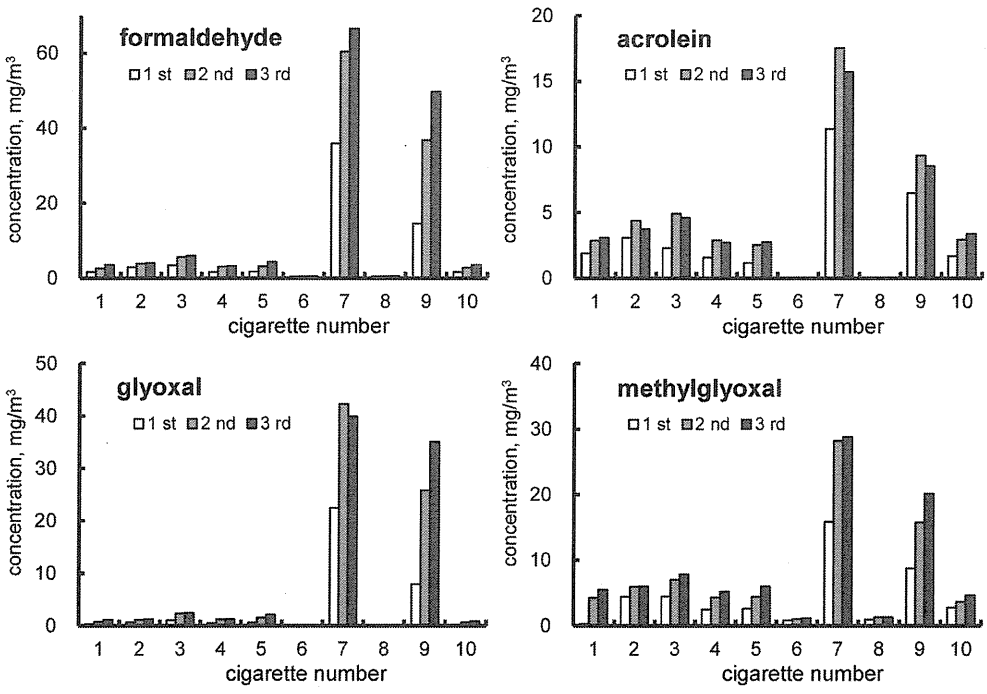

Fig. 2 Concentrations of formaldehyde, acrolein, glyoxal, and methylglyoxal generated from 10 different E-cigarettes of the same brand. Reproduced with permission from Fig. 3 in Ref. 4.