CHAPTER 2

MATERIAL AND METHOD

Instruments

1. High performance liquid chromatograph apparatus, solvent delivery system (Varian 9012Q, U.S.A.), UV-VIS detector (Varian ProStar 9050, U.S.A.) and diode array detector (Varian polychrom 9065, U.S.A.) were used with analytical column C18 150x4.6 mm i.d. and guard column C18, 5 μm to quantify formaldehyde derivative.

2. Gas chromatograph/flame ionization detector apparatus (Agilent, 6890 Series GC System, U.S.A) equipped with autosampler (Agilent, 7683 Series injector, U.S.A.) and analytical column SGE 52302 BPX5 (SGE, USA) were used for methanol and phenol quantification.

3. Gas chromatograph apparatus (Agilent, 6890 Series GC System, U.S.A) equipped with autosampler (Agilent, 7683 Series injector, U.S.A.) and MS detector (HP, 5978 mass selective detector, U.S.A.) were used for identification for methanol and phenol in air samples. The analytical column used was HP-5MS 5% phenyl methyl siloxane (J&W Scientific, U.S.A.).

4. Solid phase microextraction fiber and holder (Supelco,U.S.A.) used for extract methanol from sample sorbent tube.

5. Air sample was collected by;

   - Glass tube, filled with silica gel for phenol collection (SKC Inc., U.S.A.)
   - Glass tube, filled with XAD-7 for methanol collection (SKC Inc., U.S.A.)
   - Glass tube, filled with DNPH-coated silica gel for formaldehyde collection (SKC Inc., U.S.A.)
   - Personal sampling pump, 0.1-1.5 L/min with flexible PTFE tubing (Sensidyne Inc, USA). Calibration of air flow performed through membrane pump, Gilibrator 2 (Gilian® Sensidyne Inc., USA)

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6. Light microscope (Olympus, Japan) and cytocentrifuge, Cytospin 4 (ThermoShandon, United Kingdom) used in micronucleus assay.

(Instruments picture was shown in Appendix A)

Chemicals and reagents

All solvents used were HPLC grade. Other chemicals and reagents were all analytical grade. The detail of reagent preparation was in Appendix B.

Student population

Forty-seven dentistry students, 37 females and 10 males, who were taken human gross anatomy course, were exposed to the vapor from embalming solution during the laboratory practice for 15 weeks. Exposure time was 4 hours per week.

There is evidence that genetic risk estimation is not only based on the genetic potency of the genotoxic chemicals but different confounding factors such as age, diet, alcohol consumption and smoking habit, can also influenced the results. Therefore, when analyzing the results, these and other influential factors were taken into account in this study. Each student has been asked to complete a questionnaire a week before the class started. The questionnaire included standard demographic questions e.g. age, gender, etc., as well as medical examination e.g. number of X-ray diagnoses in the past 3 mouths and medication, etc., lifestyle e.g. smoking, coffee, alcohol, diet etc. and occupational activity such as embalming exposure (detail of questionnaire was shown in Appendix C). Students included in the study were both non- and ex-smokers. Donors were classified as non-smokers when they has never smoked or had quit smoking for more than 5 years, and as ex-smokers when they had quit smoking between 1 and 5 years ago (Pastor *et al.*, 2001). Previous to the study, all individuals had to give an informed consent. The buccal epithelial cells were obtained as described below from each student a week before class. The cells were collected again at mid and end of the semester. The interim questionnaire was also performed.

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Buccal cell collection

Collection of buccal cells was modified from Suruda *et al.* (1993). The protocol for buccal cell collection was as followed; buccal cells were swabbed from oral mucosa, left and right cheek using wood spatula. The wood spatula was immediately immersed in the vial containing 5 ml of normal saline. Three washes in normal saline were performed by centrifugation at 1,500 rpm for 10 min and the final suspension was placed directly onto a glass microscope slide in the cytocentrifuge. The slides were fixed in methanol and gracial acetic acid (3:1) for 30 min, then air dried overnight and stained with Feulgen reaction solution before counterstaining with Fast green reagent.

Micronucleus staining

Method for staining of micronucleus in buccal cells was applied from Holland (2002). The protocol for staining was as followed;

1) Placed slides in room temperature 1N HCl for 2 min and placed in 60-63°C 1N HCl for 6 min. Cooled slides were placed in room temperature 1N HCl for 2 min.

2) Slides were rinsed in distilled water for 10 min.

3) Placed slides in 0.5 % periodic acid for 20 minutes rinsed slides in running tap water for 1 min and then rinsed slides in distilled water for 5 min.

4) Placed slides in Schiff's reagent for 2-3 hours and then in warm water for 5 min.

5) Rinsed slides in running tap water for 2-3 min.

6) Air dried slides were placed in 0.5 % fast green solution for 2-3 seconds then rinsed slides three times in absolute ethanol, 2 min each time.

7) Slides were analyzed under a total magnification of 1,000x using a bright field Olympus microscope with oil immersion. When stained properly, nuclei and micronuclei should appear pinkish-red and the cytoplasm should be an emerald green. If the cytoplasm is too dark and the nucleus is not clear and distinct, destain longer in ethanol until the optimal contrast is achieved. One thousand and five hundred mononucleated buccal cells were counted. The total number of micronuclei in mononucleated buccal cells (MNBC) and frequency of mononucleated buccal cells

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with micronuclei (BCMN), in each individual were recorded. Criteria for scoring micronucleus was as followed (Holland, 2002).

- The diameter of micronucleus usually less than 1/3 diameter of the main nucleus.
- Micronucleus must be on the same plane of focus.
- Micronucleus must be the same color, texture and refraction as the main micronucleus.
- Micronucleus must have smooth oval or round shape.
- Micronucleus is not linked or connected to main nucleus.
- Micronucleus is readily distinguished from artifact such as staining particle.
- Micronucleus may touch but not overlap the main nucleus and micronuclear boundary should be distinguished from the nuclear boundary.

Metanucleated anomalies other than micronuclei, such as binucleated cells (BI), karyolysis (KL) and broken eggs (BE) were also taken into account.

Air sampling

Personal air sampler and specific sorbent tube for formaldehyde, methanol or phenol were used for air sampling. The sorbent tube breaker was used to break both ends of a sorbent tube to provide an opening at least one-half the internal diameter. This tube was used for calibrating the flow and not for collecting the sample. With flexible tubing, connected the low flow holder to the pump intake. The sorbent tube was placed into the black rubber sleeve of the low flow holder. The printed arrow on the sorbent tube shows the direction of the air flow and should point toward the pump. If there is no arrow printed on the tube, the sorbent sections should be closer to the pump. To measure the flow rate, connect the open end of the sorbent tube to an external flowmeter. Calibrate the flow rate specified in the analytical method for formaldehyde, methanol and phenol being sampled. When the flow rate has been calibrated and verified, remove the sorbent tube used to calibrate the flow (http://www.skcinc.com/aboror.asp).

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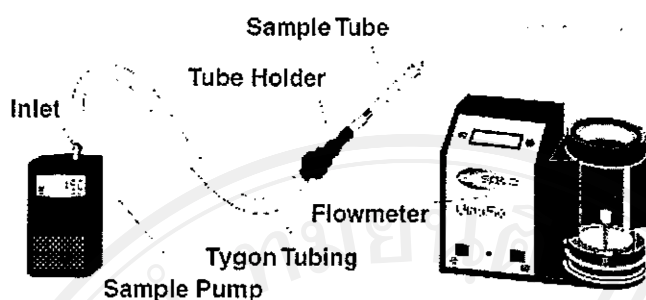

Figure 16 Personal air sampler including sample pump, sorbent tube or sample tube and a set of equipment to calibrate air sampling in this study (http://www.skcinc.com/aboror.asp).

Quantification of formaldehyde

Formaldehyde in the human gross anatomy laboratory room was analyzed based on the NISOH method 2016 (NIOSH, 1998a). The protocol was as followed;

1. Indoor air sampling

Formaldehyde exposure levels were determined using a cartridge filled with dinitrophynylhydrazine (DNPH)-coated silica gel in personal air sampler. Attached sampler to sampling pump with flexible tubing. Fifteen liters of air were sampling at 60 ml/min flow rate. Personal sampling pump was placed on a table located at the center of the gross anatomy laboratory room. Air was sampling once a week for 7 weeks and each time for 4 hours. The cartridges were stored at 4°C before analysis.

2. Sample preparation

The sorbent tube or cartridge tubes were opened by a glass-cutter. The silica gels of the front and back bed were removed separately. The dinitrophenylhydrazone (DNPHo) was extracted from silica gel by desorbing with 1 ml acetonitrile and sonicated for 30 min, stored at 4°C until HPLC analysis. In order to determine the background unexposed, blank tube were extracted and analyzed.

3. DNPHo standard preparation

Ten milliliters of concentrated hydrochloric acid was added to 2 grams of 2,4-dinitrophenylhydrazine under stirring for dissolved. Ethanol (95 %) 200 ml was added to dissolve the

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yellowish crystals mass, stirred for 30 min then filtered through Whatman No. 1 to remove undissolved hydrazine hydrochloride. Formaldehyde (40%) 0.8 ml was added to form a yellowish precipitate. Then, filtered the crystals and washed three times with 95% ethanol. The purity of DNPHo crystal was determined using differential scanning calorimeter (DSC).

4. Analysis of DNPHo

Analysis of DNPHo was carried out using HPLC and standard DNPHo calibrations curve was performed between DNPHo concentration and peak area. Standard solutions were prepared in acetonitrile for 8 concentrations (raning from 0.78 to 100 ppm) .The analyzing condition is described below;

Injection volume	: 20 µl
Mobile phase	: water:acetonitrile (55:54, v/v), 1.3 ml/min
Column	: C_{18} column, 150 x 4.60 mm (particle size 5 microns)
Detection	: 360 nm for UV-VIS detector

Quantitative analysis was performed using the following formula;

Formula 1: Calculation for DNPHo concentration

$$C_{DNPHo}\ (\mu g/ml) = (C_f + C_b) - (B_f - B_b)$$

C_{DNPHo} = concentration of hydrazone found in samples

C_f = concentration of hydrazone found in samples front

C_b = concentration of hydrazone found in samples back

B_f = concentration of hydrazone found in media blank front

B_b = concentration of hydrazone found in media blank back

Formula 2: Calculation for formaldehyde concentration

$$W\ (\mu g/ml) = C_{DNPHo} \times 30.03/210.21$$

W = concentration of formaldehyde

Molecular weight of formaldehyde is 30.03.

Molecular weight of DNPHo is 210.21.

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Formula 3: Calculation for formaldehyde concentration in air

Total mass collected (μg) = concentration of formaldehyde (μg/ml) x desorption volume

$$\text{Concentration of formaldehyde } (mg/m^3) = \frac{\text{total mass collected (mg)}}{\text{total volume of air (L)}}$$

$$\text{Concentration of formaldehyde (ppm)} = \frac{24.45 \times \text{concentration of formaldehyde } (mg/m^3)}{\text{molecular weight of formaldehyde}}$$

24.45 = molar volume of an ideal gas at 760 mm Hg and 25°C

Molecular weight of formaldehyde is 30.03.

Quantification of methanol

Methanol in indoor air of the human gross anatomy laboratory room was analyzed based on the NISOH method 2000 (NIOSH, 1998b). The protocol was as followed;

1. **Indoor air sampling**

Methanol exposure levels were determined using solid sorbent tubes equipped with personal sampling pump. Four point eight liters of air were sampling at the flow rate of 20 ml/min. Personal sampling pump was placed on a table located at the center of the human gross anatomy laboratory room. Indoor air was sampling once a week for 4 weeks and each time for 4 hours. Samples were stored at 4°C before analysis.

2. **Sample preparation**

The cartridge tubes were opened by a glass-cutter and then the silica gel of the front and back bed were removed separately. The methanol was extracted from the sorbent by desorbing with 2 ml of water and isopropanol (95:5) and sonicated for 30 min, stored at 4°C until GC analysis. In order to determine the background, unexposed blank tube were extracted and analyzed

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3. Analysis of methanol by GC-FID

Analysis of methanol was carried out using gas chromatograph and flame ionization detector (FID). The optimum condition obtained were used for determination of methanol concentration adsorbed in sorbent tube is described below;

Injection volume	: 1 μl (splitless)
Temperature	: injection, 180°C
	: column, 40°C, for 5 min
	: detector, 250°C
Carrier gas	: helium, 1 ml/min
Column	: SGE 52302 BPX5, 25 m x 220 μm (i.d.), 0.25 μm (film thickness)
Detection	: flame ionization detector

Quantitative analysis of methanol was performed using external standard calibration. Standard solutions were prepared in water and isopropanol for 7 concentrations ranging from 6.25 to 400 ppm, and then converted results to concentration of methanol in air by the following formula;

Formula 4: Calculation for methanol concentration in air

$$C\ (\mu g/ml) = (C_f - C_b) - (B_f - B_b)$$

C = concentration of methanol

C_f = concentration of methanol found in samples front

C_b = concentration of methanol found in samples back

B_f = concentration of methanol found in media blank front

B_b = concentration of methanol found in media blank back

Formula 5: Calculation for methanol concentration in air

Total mass collected (μg) = concentration of methanol (μg/ml) x desorption volume

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$$\text{Concentration of methanol (mg/m}^3\text{)} = \frac{\text{total mass collected (mg)}}{\text{total volume of air (L)}}$$

$$\text{Concentration of methanol (ppm)} = \frac{24.45 \times \text{concentration of methanol (mg/m}^3\text{)}}{\text{molecular weight of methanol}}$$

24.45 = molar volume of an ideal gas at 760 mm Hg and 25°C

Molecular weight of methanol is 32.04.

4. Analysis of methanol by GC-MS & SPME

Gas chromatograph equipped with mass spectrometer was used to identity and confirms of methanol detection in air sample. Injection sample was introduced in to the GC-MS by solid phase microextraction (SPME) technique. The SPME unit was performed manually using a SPME fiber holder. Samples were extracted with the fiber retracted pass the needle through the sample vial septum. The plunger was depressed to expose the fiber to the headspace above the sample. Analytes adsorb to the fiber in 10 minutes. The fiber was retracted into the needle and removed the needle from the sample vial. The needle was inserted into the GC injector port. The plunger was depressed to exposing the fiber in the heated zone of the injector to desorbed the analytes onto the column. The fiber was retracted and removed the needle. The analyzing condition is described below;

Injection volume	: 1 µl (splitless)
Temperature	: injection, 180°C
	: column, 40°C (5 min) to120°C (7 min) at 20°C/min
Carrier gas	: helium, 1 ml/min
Column	: HP-5MS 5 % phenyl methyl siloxane, 30 m x 250 µm (i.d.), 0.25 µm (film thickness)

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Quantification of phenol

Phenol in indoor air of the human gross anatomy laboratory room was analyzed based on the NISOH method 2546 (NIOSH, 1994). The protocol was as followed;

1. Indoor air sampling

Phenol exposure levels were determined with solid sorbent tube equipped with personal sampling pump. Fifteen liters of air were sampling at a flow rate of 60 ml/min. Personal sampling pump was placed on the table located at the center of the human gross anatomy laboratory room. Indoor air was sampling once a week for 4 weeks and each time for 4 hours. Samples were stored at 4°C before analysis.

2. Sample preparation

The sorbent tube or cartridge tubes were opened by a glass-cutter and the silica gel of the front and back bed were removed separately. The phenol was extracted from sorbent tube by desorbing with 2 ml methanol and sonicated for 30 min, stored at 4°C until analysis. In order to determine the background, unexposed blank tube were extracted and analyzed

3. Analysis of phenol by GC-FID

Analysis of phenol was carried out using a gas chromatograph and flame ionization detector (GC-FID). The analyzing condition is described below;

Injection volume	: 1 µl (splitless)
Temperature	: injection, 250°C
	: column, 80°C (1 min) to 200°C (12 min) at 10°C/min
	: detector, 280°C
Carrier gas	: helium, 1 ml/min
Column	: BPX5 , 25 m x 220 µm (i.d), 0.25 µm (film thickness)
Detection	: flame ionization detector

Quantitative analysis was performed using external standard calibration. Standard solution were prepared in methanol for 6 concentrations ranging from 0.78 to 25 ppm, and then converted results to concentration of phenol in air used the following formula;

Formula 6: Calculation for phenol concentration in air

$$C\ (\mu g/ml) = (C_f - C_b) - (B_f - B_b)$$

C = concentration of phenol

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V = volume of air sample

C_f = concentration of phenol found in samples front

C_b = concentration of phenol found in samples back

B_f = concentration of phenol found in media blank front

B_b = concentration of phenol found in media blank back

Formula 7: Calculation for methanol concentration in air

Total mass collected (μg) = concentration of phenol (μg/ml) x desorption volume

$$\text{Concentration of pheanol } (mg/m^3) = \frac{\text{total mass collected (mg)}}{\text{total volume of air (L)}}$$

$$\text{Concentration of phenol (ppm)} = \frac{24.45 \times \text{concentration of phenol } (mg/m^3)}{\text{molecular weight of phenol}}$$

24.45 = molar volume of an ideal gas at 760 mm Hg and 25°C

Molecular weight of phenol is 94.11

4. Analysis of phenol by GC-MS

To confirm the studied compound and results obtained for the real samples, GC-MS analyses were performed using a mass spectrometric detector. Samples were analyzed by GC-MS to identify the phenol. The analyzing condition is described below;

Injection volume : 1 μl (splitless)

Temperature : injection, 250°C

: column, 70°C (2 min) to 200°C (13 min) at 10°C/min

Carrier gas : helium, 1 ml/min

Column : HP-5MS 5 % phenyl methyl siloxane, 30 m x 250 μm (i.d.), 0.25 μm (film thickness)

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Data analysis

Since the numbers of micronuclei in mononucleated buccal cells (MNBC) and frequency of mononucleated buccal cells with micronuclei (BCMN) of each dentistry students were not normally distributed, the statistical significance of the change in micronuclei was assessed by Wilcoxon Sigh-rank, a nonparametric test, on the individual different scores. The associations between the MNBC and confounding factors i.e. gender, food supplement and X-ray exposure was assessed with Mann-Whitney test. The Kruskal-Wallis test was applied to detect association between the MNBC and spicy food consumption.

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