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Application of solid-phase microextraction and gas chromatography–mass spectrometry for measuring chemicals in saliva of synthetic leather workers

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ABSTRACT

Saliva is of interest as a diagnostic aid for oral and systemic diseases, to monitor therapeutic drugs, and detect illicit drug abuse. It is also attractive for biological monitoring of exposure to hazardous solvents. The major advantage of this indicator over other biological monitoring targets is that the saliva is noninvasive and less confidential in comparison with blood and urine. Salivary analysis is generally acceptable by study subjects and can be applied to investigation of a wide variety of compounds. However, very few studies have been conducted on the saliva matrix to monitor exposure to hazardous solvents. The aim of this study is to establish an analytical method, headspace solid-phase microextraction (HS-SPME) followed by gas chromatography–mass spectrometry (GC–MS), by which the saliva matrix can be monitored for multiple compounds with various polarities, such as methyl ethyl ketone (MEK), isopropyl alcohol (IPA), and *N*,*N*-dimethyl formamide (DMF) (common solvents used in synthetic leather manufacture), as well as acetone (ACE) and *N*-methyl formamide (NMF) (metabolites of IPA and DMF, respectively). We studied this technique as an alternative biological monitoring method for investigating exposure to hazardous solvents. A Carboxen/Polydimethylsiloxane (CAR/PDMS 75 μm) fiber coating was employed for this study, and various extraction and desorption parameters were evaluated. The extraction efficiency and reproducibility of analyses was improved by pre-incubation. The limits of detection were 0.004, 0.003, 0.006, 0.05, and 0.10 μg/mL for ACE, MEK, IPA, DMF, and NMF, respectively. Method validation was performed on standards spiked in blank saliva, and a correlation was made between HS-SPME and traditional solvent pretreatment methods. It was found that correlation coefficients (r) were greater than 0.996 for each analyte, with no significant differences ($p > 0.05$) between two methods. However, the SPME method achieved lower limits of detection, with good accuracy (recovery 95.3–109.2%) and precision (1.17–8.22% CV) for both intra- and inter-assay, when quality control samples were analyzed for all five compounds. The partition coefficient for each compound between the headspace of the saliva sample and the CAR/PDMS fiber coating was 90.9, 170.1, 36.4, 3.70 and 0.92 for ACE, MEK, IPA, DMF and NMF, respectively. Real sample analyses were performed on workers in a synthetic leather factory. In summary, the SPME method is a highly versatile and flexible technique for chemical measurement, and we demonstrate its application for monitoring biological exposure to hazardous solvents. Saliva monitoring using sensitive SPME approaches for determining workplace exposure should prove useful as an alternative exposure monitoring method.

1. Introduction

Personal air monitoring is a routine method used by industrial hygienists to investigate external exposure to hazardous volatile organic compounds. Total internal exposure, including topical absorption of organic solvents, can be elucidated by biological monitoring of blood and/or urinary metabolites. Many authors have produced remarkable advances in sensitive techniques which have encouraged the analysis of chemicals in conventional biological samples, particularly in urine. In comparison to blood and urine matrices, saliva has the advantages of being non-invasively collected, being readily accessible with fewer confidentiality concerns, having a much lower protein content than other physiological fluids, and being relatively free of interfering substances [1]. A good correlation between saliva and plasma levels for health investigation parameters makes saliva an attractive health diagnostic tool for systemic diseases [2]. There is a wide variety of applications in saliva investigation that are pursued by dental and medical researchers for therapeutic drug monitoring and illicit drug abuse detection. A few studies have also explored saliva from a comprehensive health perspective, considering the role of this fluid in reflecting the health, comfort, and well-being of the human organism [3].

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Some studies have studied saliva to evaluate chemical exposure and consequent health effects. For example, Ernstgård et al. investigated biological samples of exhaled air, blood, saliva, and urine in an inhalation toxicokinetics study on isopropyl alcohol and *m*-xylene exposure. The headspace of isopropyl alcohol, its metabolite acetone, and *m*-xylene were monitored in biological matrices, and the authors proposed that the compounds measured in saliva might be a useful indication of internal exposure [4,5]. Rose et al. studied concentrations of acetone in both blood and saliva during isopropyl alcohol exposure and concluded that a high correlation was found between these two biological matrices for either individual subjects or the entire study group [6]. Saliva is a complex and dynamic biological fluid. A properly established method of collection, storage, and analysis is essential to obtaining meaningful results for saliva monitoring and consequent health effect evaluation [7]. The initial goal of this research was to verify whether saliva can be included as a means of biological monitoring, providing an alternative option for exposure monitoring.

Liquid–liquid extraction followed by headspace analysis has commonly been applied for biological sample pretreatment prior to measurement [4,5]. However, liquid–liquid extraction methods suffer from complicated sample preparation procedures, sample loss, introduction of interferences, significant time consumption, and hazardous solvent consumption. The sensitivity of headspace analysis is limited, as well [8]. In contrast, solid-phase microextraction is a sample preparation method which has the unique advantages of simplicity, reliability, flexibility, and freedom from solvent consumption. This method is capable of integrating sample pretreatment and introduction into an instrument for analysis in the same device. It performs component isolation and concentration in one step, because the polymeric material coated on a fused silica fiber that acts as selective sampling media for concentrating extracted compounds, and the same polymer coated fiber can be directly introduced into the analysis instrument. As a result, the overall measurement time can be reduced [9–13]. This method has been extended to analysis of polar and non-polar volatile, semi-volatile, and non-volatile compounds in air, water, soil, natural products, and biological samples. There have been several applications using direct immersion SPME for drug analysis in saliva [1,11,14]. However, there have been few applications of HS-SPME for measuring volatiles in saliva, especially with respect to exposure monitoring.

N,*N*-Dimethyl formamide (DMF) is the most-consumed solvent for both dry and wet processes during synthetic leather manufacturing. In addition, methyl ethyl ketone (MEK), and isopropyl alcohol (IPA) are often used as solvents and co-solvents for machine cleaning and maintenance. In Taiwan, the total annual synthetic leather production is 70,000 km in length [15]. It is estimated that manufacturing 1 m of leather by the wet process requires a minimum input of 0.9 kg DMF [16]. DMF is readily absorbed into the body, either when inhaled or upon dermal contact. Exposure to DMF may cause a variety of adverse health effects, with the hepatotoxicity of DMF associated with the most relevant being the biotransformation into *N*-methyl formamide (NMF) [17]. The primary metabolic reaction of DMF is oxidation to either *N*-hydroxymethyl-*N*-methyl formamide (HMMF) by cytochrome P450 2E1 (the predominant urinary metabolite of DMF excreted in urine), *N*-acetyl-*S*-(*N*-methyl-carbamoyl)cysteine, or the minor metabolite *N*-methyl formamide. HMMF is stable in aqueous solution, but it is thermally degraded into NMF during gas chromatographic analysis. As a result, NMF is commonly employed as biological marker for exposure [18–20]. MEK is another widely used industrial chemical, and it has been studied extensively for possible human health or environmental effects. The previous studies reveal that MEK can be well absorbed by either oral or inhalation route and undergoes relatively rapid clearance in its original form from the body as a result of metabolism [21]. Isopropyl alcohol is a central nervous system depressant and a human sensitizer, and it can irritate the eyes and rhinopharynx. It is metabolized by aldehyde dehydrogenase to acetone [22].

At workplaces, multi-solvents are commonly used. The exposure of airborne organics through respiration must continuously be monitored to protect the health of workers. The consumption of DMF and co-solvents as a mixture might contribute to their adverse health effects [23]. DMF, MEK and IPA are categorized as class II toxic organic solvents under the labor safety and health law of Taiwan for preventing organic solvent toxication. While those compounds are applied in workplaces, suitable protective strategies are required, i.e., installation of ventilation systems, supply of respirators, and supply of gloves for workers to wear. In addition, personal airborne exposure monitoring at workplaces is required once every 6 months [24]. The health effects to laborers in the synthetic leather industry are a persistent cause of concern in the leather-producing industry.

The aim of this study is to establish an alternative exposure monitoring method by using solid-phase microextraction followed by gas chromatography–mass spectrometry for preparation and measurement of multi-component mixtures with different polarities in the saliva sample matrix. While similar studies have analyzed blood and urine, the study presented in this paper extended the biological monitoring to the saliva matrix. This approach is common in the therapeutic arena, but is seldom used for exposure monitoring.

2. Experimental

2.1. Materials

All reagents were analytical grade. *N*,*N*-Dimethyl formamide, isopropyl alcohol, methyl ethyl ketone and methanol were obtained from Tedia (Fairfield, OH, USA). Acetone was from Fisher Scientific (Fair Lawn, NJ, USA). *N*-Methyl formamide (>99%) and mass tuning reference perfluorotri-*n*-butylamine were purchased from Fluka (Steinheim, Switzerland). Sodium chloride was purchased from E. Merck (Darmstadt, Germany). Sodium acetate and sodium nitrate were purchased from Showa (Tokyo, Japan). Helium with 99.999% purity for GC–MS was supplied by Sanfu Gas (Taiwan, ROC). The GC injector liner and sample vials were purchased from Supelco (Bellefonte, PA, USA). The stirrer/heater with aluminum rack for sample extraction was from Barnstead/Thermolyne (SP46925, Dubuque, IA, USA). A Centrifuge was from Kubota (model 5800, Tokyo, Japan). The mechanic agitator was acquired from Scientific Industries (Vortex-Genie 2 G-560, NY, USA).

2.2. SPME fibers

The compounds investigated in this study were characterized as both semi-volatile and volatile (bp range 56–185 °C) with various degrees of polarity. The commercially available types of fiber coating with different polarities were prospectively selected for performance evaluation in the present study. The following bipolar fiber coatings on fused silica cores were included for testing: Carboxen/Polydimethylsiloxane (CAR/PDMS 75 μm), Carbowax/Divinylbenzene (CW/DVB 65 μm), Polydimethylsiloxane/Divinylbenzene (PDMS/DVB 65 μm), Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS 50 μm), and a polar coating of Polyacrylate (PA 85 μm). All SPME fiber assemblies and holders were from Supelco (Bellefonte, PA, USA).

The amount extracted with good reproducibility and low deviation was the major factor considered during fiber selection. The fiber was exposed in the headspace of the saliva sample containing

target analytes and heated at 80 °C during the 15-min extraction. At the end of the extraction period, the fiber with adsorbed analytes was immediately transferred into the modified SPME injector of the gas chromatograph and thermally desorbed, separated, and quantified. Each experiment was performed in triplicate using separate vials for extraction and analysis. The amount of analyte extracted onto each fiber type was compared under the same procedure and conditions.

2.3. Instrumentation

All analyses were performed using a solid-phase microextraction apparatus (Supelco, Bellefonte, PA, USA) coupled with a gas chromatograph (AutoSystem XL, PerkinElmer, Norwalk, CT, USA) using a fused silica capillary column (DB-WAX 60 m × 0.25 mm ID × 0.25 μm thickness of polyethylene glycol film, J&W Scientific, Folsom, CA, USA) and equipped with a quadrupole mass spectrometer (Turbo mass Spectrometer, PerkinElmer). A 0.75 mm ID liner (Supelco) was inserted into the injector for SPME fiber introduction. The GC injector temperature was 250 °C. The column oven temperature started at 40 °C, was held at that temperature for 3 min, and then was heated through the following program: 40–60 °C at 5 °C/min, 60–250 °C at 40 °C/min, and then held at 250 °C for 4 min. The carrier gas was helium with a flow-rate of 0.8 mL/min and a 1:5 split ratio. Electron impact at 70 eV was selected as the ionization mode for the mass spectrometer, and the electron multiplier voltage was 260 V. The temperature of the transfer line, ion source, and detector of the mass spectrometer were all set at 270 °C. The mass spectrometer was tuned with perfluorotri-*n*-butylamine each day on start up.

Mass spectra were used for qualitative confirmation of target analytes. For compound identification, the total mass scanning range was 30–200 *m*/*z*. The compounds were quantified in selected ion recording (SIR) mode. The target ions for quantification and confirmation were 43 and 58 *m*/*z* for acetone; 43, 57, and 72 *m*/*z* for methyl ethyl ketone; 43 and 45 *m*/*z* for isopropyl alcohol; 42, 44, and 73 *m*/*z* for DMF; and 30, 42, 58, and 59 *m*/*z* for NMF. Each target compound was identified by retention time using standards and the mass spectrum provided by the National Institute of Standard and Technology (NIST, Washington, DC, USA) spectral library. The corresponding peak areas versus concentrations were employed to construct the calibration curve of each analyte. In order to prevent column bleeding [25], a thin film, 0.25-μm thickness column was selected, as it represented a compromise between the high resolution obtained with thin films and the high capacity available with thick films.

2.4. Extraction theory and partition coefficient

HS-SPME is performed with the fiber immersed in the vapor phase above an aqueous sample [12,26,27]. Analyte equilibration occurs between the liquid sample, the headspace, and the polymeric fiber coating. At equilibrium, the amount of individual analyte initially present in the sample matrix will be distributed in three phases and can be written as Eq. (1):

$$C_0 V_s = C_f V_f + C_h V_h + C_s V_s \quad (1)$$

where C_0 is the initial concentration of analyte in the aqueous matrix, and V_s is the volume of sample. C_f, C_h, and C_s are the equilibrium concentrations of the analyte in the fiber coating, headspace, and sample, respectively. V_f and V_h are the volumes of the fiber and headspace, respectively. It is assumed that activities can be represented by concentrations. The partition coefficient between two phases is defined as the ratio of the concentration of a component in each phase, as shown in Eq. (2):

$$K_{fh} = \frac{C_f}{C_h}$$
$$K_{hs} = \frac{C_h}{C_s} \quad (2)$$
$$K_{fs} = \frac{C_f}{C_s}$$

where K_{fh}, K_{hs}, and K_{fs} are the partition coefficients of the analyte between the fiber and headspace, headspace and sample, the fiber and sample, respectively. Therefore, the mass of the analyte extracted by the fiber coating, $n_f = C_f V_f$, can be described using Eq. (3):

$$n_f = \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + K_{hs} V_h + V_s} \quad (3)$$

The amount of analyte adsorbed onto the fiber depends on the capacity of the fiber ($K_{fs}V_f$), the headspace volume ($K_{hs}V_h$), the sample volume (V_s), the thickness of the polymer coating, and the partition coefficient of the compound. SPME has a very effective concentrating effect and leads to good sensitivity. K_{fs} values usually are not sufficiently large to exhaustively extract the analyte from the matrix, hence SPME is generally considered to be an equilibrium sampling method. The amount of analyte adsorbed by the coating at equilibrium is directly related to the concentration of the analyte in the sample [12]. The mass of analyte in the system does not change significantly after each extraction [27]. Extraction time is determined by the length of time required to obtain precise extractions for the analytes with the highest partition coefficients. The partition coefficient increases with increasing molecular weight and increasing boiling point of the analyte [12]. Partition coefficient K_{fs} can be obtained by measuring both the amount extracted on the fiber and the amount in the sample matrix when the three-phase equilibrium described by Eq. (2) is reached.

2.5. Standard solution and sample preparation

A standard stock solution of saliva was prepared by spiking standards of acetone, methyl ethyl ketone, isopropyl alcohol, *N*,*N*-dimethyl formamide, and *N*-dimethyl formamide into the blank saliva collected with consent from 22 and 23 years old healthy males who had not been exposed to the target chemicals. The prepared stock standards were kept in a −70 °C freezer until use. Prior to analysis, the frozen standards and samples were defrosted.

2.6. SPME analytical procedure

An aliquot of 1 mL saliva was placed into a 15 mL amber vial with a magnetic stir bar (8 mm × 3 mm, Hong-Yu Co., Taiwan, ROC), and 0.30 g of sodium chloride salt was added. The vial was sealed with a PTFE-lined rubber septum aluminum cap and stirred at 1000 rpm and 80 °C for 180 min in a sample vial rack on the stirrer/heater for pre-incubation prior to extraction. Sample extraction onto the fiber was performed on a manual SPME holder while the fiber was inserted into the vial at a fixed depth for all analyses and exposed in the vapor phase for an appropriate period of time with constant stirring at 80 °C. The fiber never comes in direct contact with the saliva matrix, otherwise a contamination to the fiber is revealed. After extraction, the fiber was immediately exposed to the GC injector at 250 °C for desorption and analysis. All fibers were conditioned in the GC injection port prior to use according to instructions provided by the manufacturer.

2.7. Method validation

The working calibration range of the GC–MS was verified by employing optimized SPME parameters in this study for each standard analyte spiked into blank saliva matrix at five different concentrations between 0.16 and 16 μg/mL for ACE, MEK, and IPA. For DMF and NMF, the calibration range was verified for concentrations between 0.3 and 30.0 μg/mL. These calibration curves were plotted with area counts against concentrations.

Limit of detection (LoD) was performed by spiking pre-tested lowest detectable amount of each component into blank saliva matrix in seven replicates and analyzed according to established method. The corresponding concentration of three times standard deviation of seven replicates for each component was reported as LoD. Limit of quantitation (LoQ) was equivalent to three times LoD. The precision (CV%) and accuracy (recovery%) of the method were evaluated concomitantly through quality control samples of both intra- and inter-day assays while individual experiment was performed.

A parallel method validation was performed between the established HS-SPME-GC–MS method and the more traditional solvent pretreatment method. The solvent pretreatment method was performed using the same procedure as our previous study examining urine samples in our laboratory, since no specific sample pretreatment method is available for the saliva matrix [23]. In brief, the saliva sample was mixed 1:1 (v/v) with methanol by the aid of mechanical agitation for 2 min. This mixture was centrifuged at $4650 \times g$ for 10 min, and the methanol sample solution was then ready for analysis.

2.8. Study subjects

The real saliva samples were collected from workers in a synthetic leather factory. Volunteers from both the production lines workers and the administrative staffs were recruited in this study. The participants were informed orally and in written form about the design of the study and their freedom to discontinue whenever they wanted. The Ethics Review Board of China Medical University Hospital approved this study. A total of 238 valid saliva samples were collected from 34 workers twice a day at both the beginning (pre-shift) and end (post-shift) of their shift over the course of 5 consecutive days. Among them, 24 subjects were from production lines and 10 subjects were from administrative office. Some workers were off duty during the sampling period, therefore, the total number of samples was less than it should be.

For collection of saliva real samples, volunteers were asked to expectorate as much saliva as possible into a 20 mL glass vial, and the vial was sealed immediately. The collected saliva samples were kept in an icebox for temporary storage during the collection period and transferred to a −70 °C freezer in the laboratory on the same day. Real sample analysis was performed using the established SPME method.

2.9. Data processing

SAS 9.1 software for Windows was used to evaluate the results.

3. Results and discussion

There are three phases involved in the HS-SPME extraction system, namely an aqueous sample matrix, a headspace vapor phase, and a solid polymeric fiber coating. The target analytes in the saliva sample matrix were first evaporated into the gas phase, and then extracted onto the solid-phase coating of the fiber. At equilibrium, the amount of individual analyte initially present in the saliva sample matrix will be distributed in these three phases. It is necessary to optimize the analytical parameters for a SPME quantitative method because it can facilitate method applications. The conducted investigations include: (i) optimizing instrumental analysis parameters, (ii) selecting extraction method and phase of fiber coating, (iii) optimizing sample volume and vial size, (iv) optimizing extraction temperature and time, (v) verifying relevant extraction parameters, such as pre-incubation temperature and time, matrix agitation, pH, and salting effect, (vi) optimizing desorption temperatures and times, (vii) evaluating partition coefficient, (viii) validating established method quantitatively, and (ix) performing real sample analyses.

3.1. Selecting extraction method

There are two variants of SPME extraction. The first is headspace SPME (HS-SPME), in which volatile analytes transfer from the sample matrix to the headspace above the sample, and are extracted from that headspace without the SPME fiber ever directly contacting the sample. The second variant of SPME allows extraction of less-volatile, higher molecular-weight components by immersing the SPME tip in the sample matrix itself. HS-SPME is recommended for body fluid measurements [12]. The major advantage of this method in biological analysis is that it prevents direct contact of the fiber with sample, thereby eliminating contamination of the fiber surface by complex endogenous substances in the biological matrix. This limits formation of a diffusion barrier due to clotted proteins, and it also prevents burning-in of adsorbed organic material during desorption in the high-temperature GC injector port. The risk of a decreased partition coefficient, K_{fs}, between the fiber coating and sample due to depletion of the fiber coating is reduced, and the lifetime of the fiber is increased considerably because irreversible damage is delayed. The enrichment of analyte through HS-SPME is a unique advantage relative to other headspace sample pretreatment methods [12]. The HS-SPME technique also prevents GC column contamination by high-molecular-weight proteins in the biological matrix. HS-SPME was therefore employed.

3.2. Selecting phase of fiber coating

The affinity of individual compounds was different for the five types of coating, and the results showed that fibers coated with CAR/PDMS provided comprehensively better response in area counts and duplication for all the compounds studied (Fig. 1). In this figure, a logarithm transformation was performed on area counts

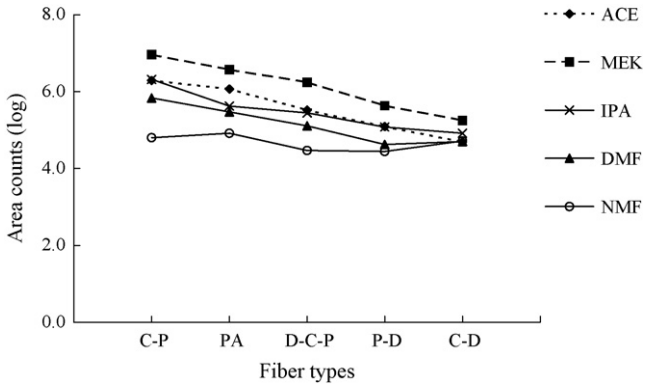

Fig. 1. The fiber performance evaluation. A logarithm transformation is performed on area counts. Fiber identifications: C-P: CAR/PDMS; PA: polyacrylate; D-C-P: DVB/CAR/PDMS; P-D: PDMS/DVB; C-D: CW/DVB.

due to large differences in response among various target compounds. However, the area counts were comparatively lower for DMF and NMF than for other, more volatile components. It could be considered that the CAR/PDMS fiber coating material is most appropriate for small and volatile compounds [28]. However, the compromise was made in fiber selection without losing precision and accuracy, even though the chemical characteristics of target analytes varies tremendously as in this study. The time for equilibration increases with fiber thickness, and thus the amount of analyte extracted onto the fiber also varied [25]. A shorter equilibrium time was preferred, and the fiber with 75 μm CAR/PDMS instead of 85 μm was therefore selected for further study. Knupp et al. analyzed *N*-hydroxymethyl-*N*-methyl formamide and *N*-methyl formamide in urine using fibers coated with PDMS/DVB [29]. In our study, the area counts of PDMS/DVB are similar to those for CAR/PDMS for NMF, but we see dramatically reduced responses for other compounds.

3.3. Optimizing sample volume and vial size

The effect of fixed sample volume with different headspace volume was investigated, and vials with volumes of 4, 15, and 20 mL were included for this study. The results showed that the 15 mL vial provided the largest area counts. This might be due to the lower headspace available for a 4 mL vial and the greater extraction time required for a 20 mL vial. Longer extraction time was not preferred, and thus the 15 mL vial was adopted in this study. In consequence, the 15 mL vial containing different saliva sample volumes of 1, 2, 4, 6, and 8 mL with corresponding headspaces of 14, 13, 11, 9, and 7 mL, respectively, were evaluated for suitable sample size. The results showed that the area count increased concomitantly with the volume of headspace. A sample volume of 1 mL with the largest headspace resulted in the largest area counts, as shown in Fig. 2, and this size was thus employed for further study.

3.4. Optimizing extraction temperature and time

SPME is an equilibrium extraction method that takes the advantage of differences in extracting phase-matrix partition coefficients to separate target compounds from interferences. This extraction technique is more selective and less susceptible to interferences [10]. In HS-SPME, the temperature will affect the partition coefficients between fiber-vapor and sample-vapor, and thus affects the amounts of analyte extracted onto the fiber. The partition coefficient between fiber and headspace, K_{fh}, decreases with increasing temperature; meanwhile the partition coefficient between headspace and sample matrix, K_{hs}, increases. On the other hand, the time for extraction is considerably reduced in HS-SPME

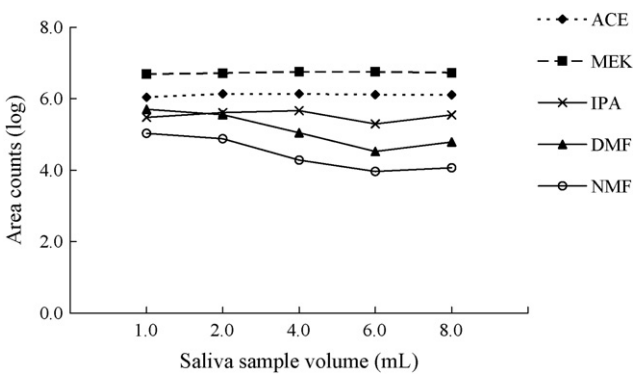

Fig. 2. The evaluation of saliva sample volumes. A logarithm transformation is performed on area counts.

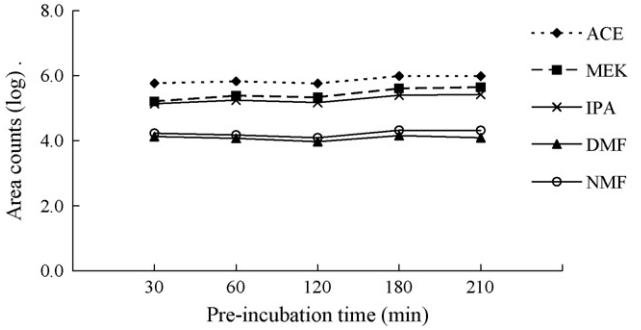

Fig. 3. The evaluation of pre-incubation time. A logarithm transformation is performed on area counts.

at increased temperatures, and an appropriate temperature control is recommended for many applications [8,12,30].

The effect of extraction temperature was studied at 30, 40, 60, and 80 °C. This study showed that an extraction temperature of 80 °C provided a response that was the most stable and comparatively higher for most compounds. Temperatures higher than 80 °C will easily form boiling bubbles which may damage the fiber due to contact with contaminants in the sample matrix.

The extraction time and the relative number of molecules extracted at a distinct time are concentration independent for the SPME sorption mechanism. However, the absolute number of molecules extracted at a particular time is linearly proportional to the concentration of analyte [12,31]. This phenomenon is the basis of SPME quantitation. An in situ headspace direct extraction was performed on saliva samples by SPME fiber at various time periods in this study. It was found that the extracted amount reached its maximum at 180 min, as revealed by the maximum instrument response with less variation compared with other time periods. However, the relative standard deviations in triplicate analyses were in a range of 10–42% for different compounds investigated.

3.5. Verifying pre-incubation temperature and time

It has been reported that the pre-incubation process prior to extraction is an important step for extraction. Ezquerro et al. proposed a pre-incubation procedure for analyzing volatile compounds in packing materials. In that procedure, the sample was preheated to evaporate the components from the sample matrix into the headspace before extraction was performed. Their study confirmed that the pre-incubation temperature and time are two important variables affecting extraction of VOCs by HS-SPME [32]. The evaluation of optimum pre-incubation temperature and time was thus performed in the present study. Different pre-incubation time periods of 30–210 min at temperatures ranging from 30 to 80 °C and various extraction time periods from 3 to 15 min were investigated. A pre-incubation temperature of 80 °C for 180 min, followed by 5 min extraction revealed overall higher area counts and good reproducibility (within 15% standard deviation) on analysis for all five compounds. There was no significant difference ($p > 0.1$) between the 180 and 210 min time periods for all analytes as evaluated through a *paired-t* test as shown in Fig. 3. The comparison was then performed between two different methods, i.e., pre-incubation for 180 min followed by 5 min headspace extraction, and in situ headspace extraction (without pre-incubation) for 180 min. The results showed that the amount extracted increased more than 50% for ACE and MEK with pre-incubation as compared with in situ extraction, and standard deviation for triplicate analyses was lower for all compounds with the pre-incubation procedure (6–15%) compared to in situ extraction (10–42%) (Fig. 4).

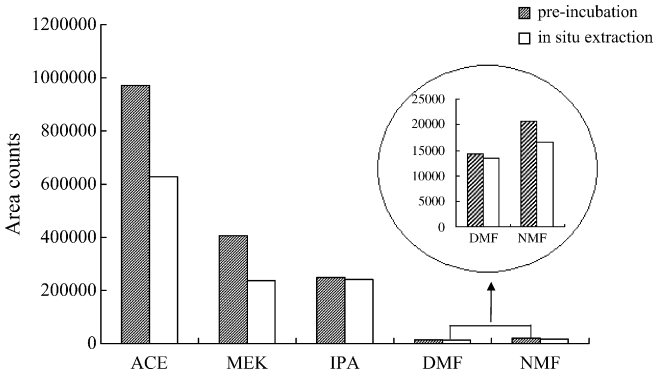

Fig. 4. Comparison between pre-incubation and in situ extraction.

The present study confirmed that the pre-incubation procedure did improve the analytical results in terms of amount extracted onto the fiber and the precision of the analysis. Our results are consistent with previous reports that pre-incubation temperature and time affect extraction efficiency and reproducibility [32]. Therefore, sample pre-incubation at 80 °C for 180 min to equilibrate the system, followed by 5 min headspace extraction was employed for further investigation. This might seem to offset the claimed time saving of SPME methods. However, the pre-incubation procedure can be processed for a batch of samples in the same time period. Therefore, the overall time consumed for analyzing a group of samples does not increase substantially, but precision and accuracy can be significantly improved. A previous studies by Avila et al. reported that the equilibration time for VOCs between the gas phase and aqueous phase in their study system was more than 200 min due to chemical and thermal equilibrium effects [30], and Poerschmann performed overnight equilibration for analytes at the ppb level [27]. The pre-incubation time employed in our study is acceptable when compared to those in previous papers.

3.6. Investigating matrix agitation

Among the three phases involved in the headspace extraction, the diffusion coefficient of volatile compounds in the gas phase is very large. As a consequence, the mass transfer of analytes from headspace to the fiber is fast. The extraction rate for the HS-SPME therefore depends on the mass transfer efficiency from the sample matrix to headspace. Magnetic stirring of the aqueous matrix can improve the extraction rate significantly [10]. The saliva sample matrix was viscous which is not favorable for improved mass transfer efficiency. However, sample agitation will speed up the mass transfer of analytes from the saliva matrix to the headspace and assist in system equilibration. As a result, the extraction rate is increased [28]. The rate of magnetic stirring was therefore varied in the study system, and extraction efficiency was evaluated. Magnetic stirring revolution rates of 800, 1000, and 1200 rpm were investigated, and it was found that the rate of 1000 rpm provided the highest efficiency in reaching equilibrium, with area counts being quite precise for all components. Ultrasonic agitation was also evaluated but was found to be inferior to magnetic stirring. This result agrees with previous reports that magnetic stirring was better than sonication for the detection of clozapine in plasma, and it was concluded that magnetic stirring was better applied for SPME in biomedical analysis [12].

3.7. Investigating pH and salting effect

The extraction efficiency of water-soluble compounds can be affected either by adjusting the pH or by modifying the ionic strength. Adding sodium chloride to the sample or changing the sample pH prior to extraction can increase the ionic strength of the solution and, in turn, reduce the solubility of analytes in solution. The amount of analyte extracted by the fiber can thus be increased [28]. In the present study, pH adjustment and salt addition were investigated. The pH of saliva for healthy people is in the range of 6.8–7.1, with no change during storage at −80 °C for 6 weeks [33]. The pH of a pooled blank saliva matrix was measured to be 6.9 in this study. The effect of pH on sample preparation was tested at three different values of pH 4.0, 6.9, and 10.0. The pH of saliva matrices at pH 4.0 and 10.0 was adjusted by either acidic or basic buffer solutions. The results revealed that there was no significant difference among pH 4.0, 6.9, and 10.0 in terms of instrument responses and variations. Saliva samples were analyzed with no adjustment on pH, therefore. The near-neutral sample matrix limits the possibility of damage by base or acid in the gas phase.

Adding salt to the aqueous sample matrix will change its electrolytic properties, and thus affect the solubility of analytes [9]. The amount and type of salt added was evaluated. NaCl, $NaNO_3$, CH_3COONa were evaluated. A saturated amount of each salt was added into the same saliva sample matrix individually and studied. NaCl provided highest area counts and the least deviation (especially for DMF and NMF) relative to samples containing $NaNO_3$, CH_3COONa, or lacking salt. The responses of acetone and isopropyl alcohol did not increase as much as those of DMF and NMF. The MEK peak area did not change significantly.

Different amounts of NaCl at 0.10 g (10%, w/v), 0.20 g (20%), and 0.30 g (30%) were added into the saliva sample matrix. The results showed that the saturated amount of 0.30 g of NaCl increased the area counts about 30% for ACE and IPA, by more than 140% for DMF and NMF, but yielded no obvious increase for MEK (Table 1). The addition of 0.30 g NaCl was employed for further study. The amount added in this study was in agreement with the recommendation of 25–30% salt addition to increase ionic strength of the aqueous layer and drive the organic components away from saliva matrix into the vapor phase that leads to an increase of the extraction efficient in HS-SPME because of salting-out effects [9,27]. The present study confirmed that the extraction efficiency was increased by addition

Table 1
The effect of salt on area counts.

Analyte	Amount of NaCl added (%, w/v) (n = 3)			
	0	10	20	30
Acetone	729,149 (3.55)	729,133 (4.28)	802,470 (6.42)	948,559 (1.96)
Methyl ethyl ketone	4,100,320 (2.68)	3,964,510 (1.56)	4,212,779 (7.22)	4,224,053 (4.24)
Isopropyl alcohol	345,538 (5.71)	229,811 (5.30)	264,842 (3.26)	440,407 (3.16)
N,*N*-Dimethyl formamide	350,353 (7.88)	408,536 (4.33)	400,962 (2.22)	839,462 (1.61)
N-Methyl formamide	91,519 (10.57)	88,571 (1.08)	96,821 (12.31)	261,591 (4.61)

Value: average area counts (CV%).

of salt, however, the pH value did not show a significant effect on the extraction.

3.8. Optimizing desorption temperatures and times

After analytes were extracted onto the fiber, it was immediately transferred to the GC injection port for desorption. The optimum desorption temperature was evaluated to make sure that desorption temperature was high enough to efficiently desorb extracted analytes and prevent carryover between samples. Also, the temperature should be low enough to avoid depletion of fiber coating. Various temperatures from 220 to 250 °C were investigated and the results showed that the highest area counts and lowest deviation occurred for a desorption temperature of 250 °C. This coincides with the factory-recommended operating temperature of the fiber. Fiber desorption at 250 °C was thus used for further study.

The desorption time will affect the desorption efficiency as well as the potential for carryover and fiber coating depletion. Desorption times of 1, 2, 3, 5, 8, and 12 min were evaluated for each analyte at 250 °C. At a time period of 3 min, all the compounds were desorbed efficiently with best response and least deviation. A desorption time of 3 min was employed for the following study. The desorption efficiency for each analyte was then evaluated at a temperature of 250 °C for 3 min, and the recoveries were 98.8%, 99.8%, 97.2%, 98.8%, and 95.3% for acetone, methyl ethyl ketone, *iso*-propyl alcohol, *N*,*N*-dimethyl formamide, and *N*-methyl formamide, respectively.

3.9. Evaluating partition coefficient

The partition coefficient for each component was measured through the calibration curve established using the instrument response versus mass for each component. The mass extracted onto the fiber and in the aqueous sample matrix was measured concomitantly. The partition coefficients obtained in the present study were 90.9, 170.1, 36.4, 3.70, and 0.92 for ACE, MEK, IPA, DMF, and NMF, respectively. The variations in peak area on the chromatogram were large for any given individual analyte. The area counts of DMF and NMF significantly differed from those of ACE, MEK, and IPA. As a consequence, order-of-magnitude differences were calculated in the partition coefficients for the various analytes. This result might be due to the formation of hydrogen bonds of NHR groups for DMF and NMF that in turn resulted in depressed vapor pressure and lower partition coefficient. As a rule of thumb, analytes with a molecular mass below 200 g/mol and (or) without groups forming hydrogen bonds, i.e., NRH groups, NH_2 groups, and OH groups, are most suitable for HS-SPME because they are likely to have a high-vapor pressure [12]. Our results revealed that partition coefficients decreased with increasing polarity of compounds. In order to investigate whether the partition coefficient varied between high and low concentrations, different concentrations of 9.5–95.0 μg/mL for DMF and NMF, and 7.8–78.5 μg/mL for ACE, IPA, and MEK were assessed. The results showed that the relative standard deviation of partition coefficient for all analytes was in the range of 2.1–15.0%. It could be confirmed that the extraction process is reproducible under the present system, and the saliva samples could be quantitatively measured for multiple components. Our results were in agreement with Dugay et al. who studied atrazine along and mixture of other eleven pesticides at ppm concentrations; they concluded that the partition process was reproducible and the SPME extraction method was quantitative [28]. The amount of analyte adsorbed onto the fiber material at equilibrium in HS-SPME is directly related to the concentration of the analyte in the vapor phase and thus in the aqueous sample matrix.

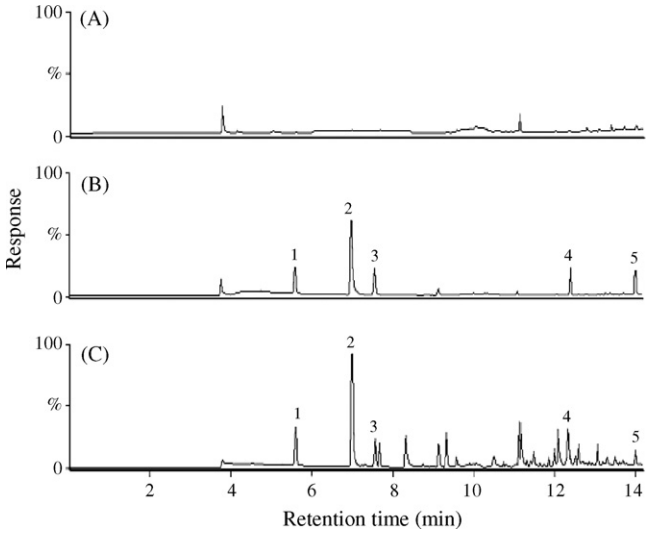

Fig. 5. Chromatograms of saliva samples: A: blank; B: standards in blank saliva matrix; C: real saliva sample. Peak identifications: 1, acetone; 2, methyl ethyl ketone; 3, isopropyl alcohol; 4, *N*,*N*-dimethyl formamide; 5, *N*-methyl formamide.

3.10. Validating established method

The chromatogram of the established SPME-GC–MS method is shown in Fig. 5. The calibration curve of all target compounds in saliva matrix under the optimized analytical conditions was constructed. The linear regression coefficients (r) were greater than 0.997 for all compounds. The limits of detection were 0.004, 0.003, 0.006, 0.05, and 0.10 μg/mL for ACE, MEK, IPA, DMF, and NMF, respectively. For quality control assessment of the method, both intra- and inter-day assays were performed at two different concentrations within the calibration ranges of analytes. The overall average precision (CV%) was in a range of 1.17–8.22%, and accuracy (recovery%) was in a range of 95.3–109.2% through the analysis of quality control samples (Table 2).

The parallel method validation between HS-SPME and solvent pretreatment was evaluated for five target compounds in saliva matrix. Three different concentrations within the calibration range for each compound were statistically evaluated by *paired-t* test, and these resulted in no significant difference ($p > 0.05$) with correlation coefficients, r, in the range of 0.995–0.999 for all five compounds. In comparing the MDL between the two methods, the SPME method provided approximately 2–70 times lower limits than that of the solvent pretreatment method. Our results confirmed that sensitivity of the measurement can be improved, and a lower range of sample concentrations can be detected by the HS-SPME method.

Table 2
Precision and accuracy of HS-SPME method for saliva.

	Analyte				
	ACE	MEK	IPA	DMF	NMF
Intra-assay precision (CV%)					
QC1	2.90	3.02	4.41	1.44	5.10
QC2	4.72	1.17	4.82	4.94	4.72
Inter-assay precision (CV%)					
QC1	7.75	4.75	8.12	6.82	8.22
QC2	6.55	3.89	5.20	7.11	4.15
Overall recovery (%)					
QC1	105.6	97.7	101.1	103.9	109.2
QC2	101.1	95.7	97.3	100.4	95.3
LoD (μg/mL)	0.004	0.003	0.006	0.05	0.10

Table 3
Results on real sample analyses.

Analyte	Work shift	Range (μg/mL)	Median (μg/mL)	Geometric mean[a] (μg/mL)	No. positive samples (%)
ACE	Pre-	ND*–7.03	0.41	0.48	117 (98)
	Post-	ND*–7.97	0.54	0.51	117 (98)
MEK	Pre-	ND*–11.16	0.21	0.33	99 (83)
	Post-	ND*–12.07	0.33	0.36	104 (87)
IPA	Pre-	ND*–22.13	1.01	1.28	113 (95)
	Post-	ND*–28.17	1.31	1.51	116 (97)
DMF	Pre-	ND*–24.70	0.77	2.03	80 (67)
	Post-	ND*–31.43	1.40	2.48	99 (83)
NMF	Pre-	ND*–31.74	0.08	5.16	39 (33)
	Post-	ND*–41.86	3.84	9.58	71 (60)

ND*: result < limit of quantitation (LoQ).
[a] Sample amounts less than LoQ were not included.

The concentrating effect results from partitioning the analyte into the fiber from the gas phase, and therefore the sensitivity can be substantially increased [9].

Under normal operation in the present study, each fiber can be used more than 100 times without loss of precision or accuracy, as measured by the analytical results on standards within the quality control criteria (accuracy >85%, CV <15%). Our result was comparable with previous reports by Dugay et al. who claimed that fibers could be re-used for analysis of drinking water, surface water, and run-off from 27 to more than 100 times, depending on the sample matrix [28]. Salt plugging of the sheath protecting the SPME fiber may become a problem when salt is added to enhance extraction efficiency. As a result, the fiber coating might be scratched and damaged. Procedures preventing the deposition of salt must be incorporated during analysis, such as avoiding matrix splashing during sample agitation, and using proper solvent for fiber cleanup after each experiment. The method blank sample was checked for a cycle of 20 runs to ensure that carryover was not present.

Saliva sample storage stability was also investigated. The blank saliva matrix spiked with standards at the highest and middle concentrations of the calibration range were evaluated to determine the duration of sample stability. Samples were stored in a −70 °C freezer for 28 days. Samples containing either high or low concentrations were studied in triplicate on days 1, 7, 14, 21, and 28. The average recoveries relative to the first day samples were calculated, and it was found that the overall recoveries were greater than 86.9%. Our results suggest that a storage period of 28 days or less is recommended for saliva samples.

Matrix effects on sampling efficiency were evaluated by analyzing standards of the study compounds in reagent water and saliva matrix individually. The instrument responses between the two methods for each compound were statistically compared by *paired-t* test. The results showed that there was no significant difference for any of the five compounds ($p > 0.1$) when comparing the two methods over the calibration range of the SPME method. This indicated that there was no significant matrix effect on extraction efficiency and analytical measurement for the established method.

3.11. Performing real sample analyses

Organic solvents also have variable lipophilicity and volatility. These properties, coupled with small molecular size and lack of charge, make inhalation the major route of solvent exposure and provide for ready absorption across the lung, gastrointestinal tract, and skin. Through external and internal respiration, the exchange of respiratory gases between the air and the blood, and between the blood and the body cells, occurs by diffusion [34]. By passing through the capillary wall, the basement membrane, and the membrane of the glandular epithelial cells, compounds circulating in blood can be discharged into the salivary duct. Passage of the chemical through the lipophilic layer of the epithelial membrane is the rate-determining step. It is implied that only compounds with a certain degree of lipophilicity can accomplish this passage. Salivary chemical concentrations generally reflect the free fraction of the chemical in blood [3]. In the present study, the observation of NMF and ACE in saliva, the metabolites of inhaled organic vapor of DMF and IPA, respectively, might be due to the same mechanism as the appearance of drug in saliva.

The results of real sample analyses showed that the concentrations in saliva ranged from being non-detectable (ND) to 7.97 μg/mL, ND to 12.07 μg/mL, ND to 28.17 μg/mL, ND to 31.43 μg/mL, and ND to 41.86 μg/mL, for ACE, MEK, IPA, DMF, and NMF, respectively. Samples identified as ND were below limits of quantitation. The geometric mean of saliva samples was higher at the end of workers' shift than those at the beginning of the workers' shift for all five compounds. Total number of positive samples for all compounds investigated in 119 valid samples in both pre-shift and post-shift were in the range of 39–117 (33–98%) (Table 3). Correlations between concentrations in airborne and saliva samples for health effect evaluation might be established and will be published elsewhere.

4. Conclusions

The SPME method is compatible with GC–MS by selecting an appropriate polarity and thickness of the fiber coating material and maintaining a consistent extraction time and temperature parameters. The method can provide reproducible and quantifiable results for matrix matched sample systems over an appropriate concentration range for measuring multiple components with different polarities. The linearity can be extended over a wider range of concentration by setting suitable analytical parameters, the detection limits are lower than those of the solvent pretreatment method for all studied compounds, and SPME method can be applied to measure small sample volumes. The precision and accuracy of the method were similar to those obtained using traditional solvent pretreatment. The pre-incubation step for sample extraction can be operated as a batch with a large number of samples, and the average time for individual sample analysis should be acceptable. SPME is based on the equilibrium between matrix and extracting phase, and that in turn makes the method more selective, less susceptible to interferences, and suitable for analysis of complex biological sample matrices, such as saliva. Quantitative measurement can be

performed by calibration on matrix-matched spiked standards and then using those parameters for multi-component unknown sample extraction and analysis. The successfully established method in this study for multiple compounds with different polarities in a saliva matrix could provide an alternative option to extend the scope of biological monitoring.

Conflict of interest

There are no competing interests.

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References

[1] B.J. Hall, M. Satterfield-Doerr, A.R. Parikh, J.S. Brodbelt, Anal. Chem. 70 (1998) 1788.
[2] M.W.J. Dodds, D.A. Johnson, C.-K. Yeh, J. Dent. 33 (2005) 223.
[3] J.K.M. Aps, L.C. Martens, For. Sci. Int. 150 (2005) 119.
[4] L. Ernstgård, B. Sjögren, M. Warholm, G. Johanson, Toxicol. Appl. Pharmacol. 193 (2003) 147.
[5] L. Ernstgård, B. Sjögren, M. Warholm, G. Johanson, Toxicol. Appl. Pharmacol. 193 (2003) 158.
[6] D.M. Rose, A. Muttray, O. Mayer-Popken, D. Jung, J. Konietzko, Eur. J. Med. Res. 4 (1999) 529.
[7] R.G. Schipper, E. Silletti, M.H. Vingerhoeds, Arch. Oral Biol. 52 (2007) 1114.
[8] J. Pawliszyn, Trends Anal. Chem. 14 (1995) 18.
[9] Supelco Bulletin 923, Solid Phase Microextraction: Theory and Optimization of Conditions, Sigma–Aldrich Co., 1998.
[10] J. Pawliszyn, Trends Anal. Chem. 14 (1995) 113.
[11] F.M. Musteata, J. Pawliszyn, J. Pharm. Biomed. Anal. 37 (2005) 1015.
[12] S. Ulrich, J. Chromatogr. A 902 (2000) 167.
[13] J. Pawliszyn, Solid-Phase Microextraction: Theory and Practice, Wiley-VCH, New York, 1997, p. 16.
[14] A.C.D. Lucas, A. Bermejo, P. Fernandez, M.J. Tabernero, J. Anal. Toxicol. 24 (2000) 93.
[15] The Annual Report of Synthetic Leather Manufacturers Union on Taiwan, Synthetic Leather Manufacturers Union (SLMU), Taipei, Taiwan, 2006.
[16] C.H. Lu, L.S. Lai, Ind. Pollut. Control Taiwan 29 (1989) 102.
[17] P.G. Pearson, A. Gescher, E.S. Harpur, Biochem. Pharmacol. 36 (1987) 381.
[18] J. Mráz, F. Tureček, J. Chromatogr. 414 (1987) 399.
[19] P. Kestelle, A.P. Gledhill, M.D. Threadgill, Biochem. Pharmacol. 35 (1986) 2283.
[20] J. Mráz, H. Cross, A. Gescher, M.D. Threadgill, J. Flek, Toxicol. Appl. Pharmacol. 98 (1989) 507.
[21] G.D. Strickland, Environ. Health Perspect. 101 (1993) 566.
[22] IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 71, 1999.
[23] V.-S. Wang, T.-S. Shih, C.-C. Cheng, H.-Y. Chang, J.-S. Lai, C.-C. Lin, J. Occup. Environ. Med. 46 (2004) 729.
[24] Guidelines Governing Prevention of Organic Solvent Toxication, Council of Labor Affairs, Executive Yuan, Taiwan, ROC, 2003.
[25] P.M. San Juan, J.D. Carrillo, M.T. Tena, J. Chromatogr. A 1139 (2007) 27.
[26] R.U. Holt, J. Chromatogr. A 937 (2001) 107.
[27] J. Poerschmann, Z. Zhang, F.-D. Kopinke, J. Pawliszyn, Anal. Chem. 69 (1997) 597.
[28] J. Dugay, C. Miège, M.-C. Hennion, J. Chromatogr. A 795 (1998) 27.
[29] V.F. Knupp, E.M.A. Leite, Z. de Lourdes Cardeal, J. Chromatogr. B 828 (2005) 103.
[30] M.A.S. Avila, R. Breiter, H. Mott, Chemosphere 66 (2007) 18.
[31] B. Shurmer, J. Pawliszyn, Anal. Chem. 72 (2000) 3660.
[32] Ó. Ezquerro, B. Pons, M.T. Tena, J. Chromatogr. A 963 (2002) 381.
[33] E. Silletti, M.H. Vingerhoeds, W. Norde, G.A. van Aken, Food Hydrocolloids 21 (2007) 596.
[34] S.E. Gunstream, Anatomy and Physiology with Integrated Study Guide, 2nd ed., The McGraw-Hill Companies Inc., NY, 2000, p. 285.