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Quantitative Determination of Volatile Organic Compounds using HS-SPME/GC-MS: A Comprehensive Reviewed Application on Meat

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Abstract: Headspace-Solid Phase Microextraction (HS-SPME) in combination with Gas Chromatography-Mass Spectrometry (GC-MS) is currently used for identification of Volatile Organic Compounds (VOCs) in food and environmental samples. Although, HS-SPME is a reliable technique with good sensitivity and selectivity, several factors must be thoroughly considered for accurate and precise quantification of VOCs. This study focuses on the state of the art review for quantitative determination of VOCs using the same in both air and water samples. Selections of appropriate techniques for generating external calibration in conjunction with the experimental findings on meat VOCs are also presented. A simple laboratory set up was designed for generation of calibration curves for ethanol and acetic acid and statistically significant reproducibility were obtained for the same.

Key words: VOCs, meat, HS-SPME/GC-MS, calibration, samples

INTRODUCTION

Salmonella typhimurium in meat (beef) is a critical concern since, Salmonella is the second leading cause of food-borne illness worldwide. There is a need to develop rapid and reliable methods for determination of the status of Salmonella in beef to ensure consumer safety. Multidisciplinary projects are underway in The Bioimaging and Sensing Centre, Department of Agricultural and Bio-Systems Engineering, at North Dakota State University (NDSU) to develop hand-held intelligent sensors to enable rapid detection of Salmonella contamination in meat, based on olfactory sensing of headspace gases. The overall aim of this endeavor is to develop these hand-held sensors for consumers to provide them information about the status of the packaged meat, prior to consumption. In the studies, manual Headspace-Solid Phase Microextraction (HS-SPME) in combination with Gas Chromatography-Mass Spectrometry (GC-MS) is being used for the identification of the volatile compounds in the headspace of beef samples. Several techniques used for quantifying the Volatile Organic Compounds (VOCs) using SPME have been discussed here. A review of the state of the art techniques of calibration of VOCs using SPME was carried out to aid us in the research of quantification of VOCs in the headspace of meat samples. Based on the review, a method has been developed for identification of the same in Salmonella contaminated beef samples.

Basic principle of SPME: The technique of Solid-Phase Microextraction (SPME) dates back to 1989 when it was introduced by Belardi and Pawliszyn (1989) and Arthur and Pawliszyn (1990) to redress limitations inherent in Liquid-Liquid (LLE) and Solid-Phase (SPE) extractions. SPME is a commercially available, fast, simple, sensitive, solvent-free, reusable and cost-effective sampling and analysis technique. SPME integrates sampling, extraction, concentration and sample introduction into a single solvent-free step. Analytes in the sample are directly extracted and concentrated on the extraction fiber. SPME can also be coupled to High Performance Liquid Chromatography (HPLC) or HPLC/MS to analyze weakly volatile or thermally labile compounds not amenable to analysis by GC or GC/MS. These hyphenated techniques have been widely used to analyze Volatile Organic Compounds (VOCs) in food and beverages, in clinical biochemistry, plant biology and in environmental samples of air, water and soil (Zhang and Pawliszyn, 1993; Vas and Vekey, 2004).

The SPME apparatus has the appearance of a modified syringe, consisting of a fiber holder and a

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retractable fiber (1-2 cm long). The SPME fiber is a thin fused silica-optical fiber coated with a thin polymer film such as Polydimethylsiloxane (PDMS), Divinylbenzene (DVB), Carbowax (Carboxen) or combinations of these. There are two typical SPME applications sampling gases/Headspace (HS) and solutions. In either case, the SPME needle is inserted into the appropriate position through a septum in the headspace, the needle protecting the fiber is then retracted and the fiber is exposed to the environment. The polymer coating acts like a sponge, concentrating the analytes by absorption/adsorption processes. Post sampling, the fiber is retracted into the needle and then desorbed into the HPLC or GC injection ports. Prior to GC/MS applications, the fiber is conditioned in the GC injector port at the desorption temperature for 0.5-4 h using high-purity carrier gases (Pawliszyn, 1999; Vas and Vekey, 2004).

Types of SPME: In Headspace (HS-SPME), the fiber is exposed to the vapor phase above a gaseous, liquid or solid sample. In Direct Immersion (DI-SPME), the fiber is directly immersed into the sampling solutions. If the sample is agitated with a magnetic stirrer or ultrasonicated, the equilibrium is reached faster.

Theory of HS-SPME: The kinetics of SPME is better understood by examining a three-phase system in which liquid polymeric coatings, headspace and aqueous solutions are involved. The amount of analytes absorbed by the liquid polymeric coating is related to the overall equilibrium of analytes in the 3-phase system. Since, the total amount of an analyte should be the same during extraction, researchers have (Zhang and Pawliszyn, 1993):

$$C_0V_2 = C_1V_1 + C_2V_2 + C_3V_3 \quad (1)$$

Where:

C_0 = The initial concentration of the analyte in the aqueous solution

C_1, C_2 and C_3 = The equilibrium concentrations of the analyte in the coating, the aqueous solution and the headspace, respectively

V_1, V_2 and V_3 = The volumes of the coating, the aqueous solution and the headspace, respectively

If coating/gas partition coefficient is $K_1 = C_1/C_3$ and gas/water partition coefficient is $K_2 = C_3/C_2$, then the amount of analyte absorbed by the coating (capacity of the coating) can be expressed as:

$$\begin{aligned} n &= (C_0V_1V_2K_1K_2)/(K_1K_2V_1 + K_2V_3 + V_2) \\ &= (C_0V_1V_2K)/(KV_1 + K_2V_3 + V_2) \end{aligned} \quad (2)$$

where, $K_1K_2 = K$ (Zhang and Pawliszyn, 1993). Equation 2 describes the mass of analyte absorbed by the fiber coating at equilibrium. SPME is an equilibrium process and at equilibrium, the concentration of the analyte in the fiber coating is directly proportional to its concentration in the headspace. Because the volume of the fiber coating is constant in fibers, the coating volume need not be included in calibration calculations. The theory simplifies to the absolute amount of analyte absorbed by the fiber and is directly proportional to headspace concentration at equilibrium.

Although, SPME has maximum sensitivity at the equilibrium point, full equilibrium is not necessary for the purpose of identification of volatiles and also for accurate and precise analysis because of the linear relationship between the amount of analyte absorbed by the SPME fibre and its initial concentration in the sample matrix under non-equilibrium conditions. Thus, ability to use HS-SPME quantitatively before equilibrium is reached permits shorter sampling times producing a fast, economical and versatile technique for analyzing VOCs (Zhang and Pawliszyn, 1993; Pawliszyn, 1997).

Need for calibration for quantitative information: SPME has obvious utility for qualitative analysis of natural volatiles from plants, fungal cultures and other sources however, quantification of these volatiles is not a trivial task. The SPME fibers are not uniformly sensitive to all compounds and therefore relative GC peak areas for a SPME sample do not properly reflect the true proportions of the components in the headspace. Besides, factors such as sampling time and temperature can affect quantification results. These make it imperative to generate standard calibration curves for the VOCs detected for evaluation of their respective headspace concentrations directly from GC peak area responses.

Types of calibration: The decision on choice of quantification approach using SPME depends on the sample matrix, its complexity and the Extraction Method used. There are three common approaches of calibration-external calibration, internal standard comparison and the method of standard addition.

External standard calibration method is the most commonly used method. It has been stated that in external standard Calibration Method, the analyte for which quantitative information is desired, standard solutions of the analyte are prepared over a range of concentrations (expected in the sample) and each solution is analyzed using SPME-GC/MS. A calibration curve is constructed with concentration and peak area response and a comparison of the detector response of the sample extract

to the same determines the amount of analyte in the unknown sample. This technique is applied for simple sample matrices such as gaseous or liquid samples (HS of drinking water) that do not have interferences or high levels of organic solvents and for homogenous samples that do not vary in the type and total number of compounds that are present (Spme Supelco, 2001a).

Internal standard calibration has been defined as the technique which requires the addition of a known amount of a known analyte into the calibration standards and samples. It is critical that internal standards are similar in analytical behavior to the target analytes but should not be present in the sample. The ideal internal standard is an isotopically labeled analogue of the analyte of interest, e.g., toluene-d_8 for toluene and other similar volatile aromatics. This approach helps compensate sample to sample variations in extraction and desorption caused by the sample matrix. This technique is applied to complex gaseous or liquid mixtures or complex solid samples (though not recommended for highly complex matrices such as protein, fat or humic material) which can be dispersed in a liquid and headspace sampling performed (Spme Supelco, 2001a; Vas and Vekey, 2004).

The standard addition approach has been defined as the approach which involves spiking the sample matrix with known concentrations of the target analyte which originally has an unknown concentration of the same. The mixtures are analyzed. A plot of peak area response against concentration is constructed for a range of concentrations for the target analyte. Extrapolation of the plot to zero response gives the original concentration of the target analyte in the unspiked sample. This calibration method is rarely used because of the need for extensive sample preparation, particularly if the number of the target analytes is large (Spme Supelco, 2001a; Vas and Vekey, 2004; Ouyang and Pawliszyn, 2006).

Factors that affect precision of quantitative calibration using SPME: The most important factors that affect quantitative calibration using SPME are agitation, temperature, sampling time, sample matrix components (pH, salt concentration), sample volume, headspace volume, vial shape and size, fiber positioning during injection, analyte losses, moisture in the needle, geometry of the fiber and conditioning of the fiber coating. The details of how these factors affect precision in SPME are given by Pawliszyn (1999) and by Spme Supelco (2001b).

Different quantitative calibration techniques reported for VOCs from various samples: The headspace gases (gaseous metabolites of microorganisms) of meat are generated in trace quantities. Quantification techniques for determination of trace VOCs in food and environmental samples are presented below. These would provide an insight for design of procedure for measurement of VOCs in meat samples.

Environmental samples (Air/Gas samples)

Using sampling bulbs/Chambers of different volumes: To evaluate VOCs in indoor air, SPME was conducted using a 75 μm PDMS/Carboxen fiber and for analytes such as acetaldehyde, acetone, benzene, cyclohexane, trichloroethylene, toluene, butyl acetate, p-xylene, α-pinene and n-decane with external calibration approach. A specially designed standard gas generating device was used for continuous injection of liquid VOCs into a controlled airflow wherein the compounds were vaporized. Successive air dilutions of concentrated standard gases were then applied to reach the desired concentrations. The sampling chambers (glass bulbs) of different volumes (250, 375 and 1000 mL) equipped with teflon stopcocks and septum for SPME fiber introduction were used. Sampling was realized in static mode (stagnant air) (Larroque *et al*., 2006) for 30 min to 17 h.

Using round bottom flasks: Calibration has been attempted using simple 5-neck bottle (5.5 L) in estimation of α-pinene in wood with provisions for temperature and humidity control. Difference in humidity was created by varying concentrations of saturated salt solutions placed at the flask bottom. α-pinene was placed in the flask; after 1 h of equilibrium time, HS-SPME was carried out for 10 sec to 2 h. Calibration curves were generated at different extraction temperatures of 24, 40, 70 and 100°C (Rupar-Gadd *et al*., 2006).

Using tedlar bags and tedlar sampling bulbs: For quantitative determination of VOCs in ambient workplace air, analytical gas standards were prepared using volumetric injection. A known amount of TO-14 standard gas mixture was loaded into 1 L tedlar bag through a septum using a gas-tight syringe. After sample preparation, SPME syringe needle was inserted into a Tedlar bag. The fiber was then inserted into the bag and the extracted gases analyzed by GC/MS. Extraction time profiles at 10 ppbv were constructed for benzene, toluene, ethylbenzene, m, p and o-xylene at 26°C (Lee *et al*., 2002). These sampling bulbs have been also been used to quantify alkanes and aromatic hydrocarbons in human breath (Hao *et al*., 2005).

Using standard gas generators and permeation tubes: In this method, standard gas-sampling device (Kin-Tek standard gas generator) and certified permeation tubes

were used to generate standard gas concentrations of formaldehyde and BTEX (benzene, toluene, ethylbenzene and xylenes), typical of indoor and outdoor air. The sampling chamber provided steady-state mass flow of these gases at constant temperature and SPME was conducted for 10 sec to 12 min at 21-25°C for quantification of the same (Koziel *et al*., 2001; Tumbiolo *et al*., 2005).

Using specially designed flow-through sampling chamber: Calibration of VOCs in indoor and outdoor air have been reportedly carried out using standard gas generating device with flow-through sampling chambers to provide a wide range of target VOC concentrations at constant temperatures. A special air sampling system consisting of a long glass cylinder with four different diameters was constructed and installed downstream from the standard gas generator. PDMS/Carboxen fiber was exposed for 5-60 sec in sampling port and the air temperature in the main sampling chamber was varied from 22-40°C for extractions (Koziel *et al*., 2000).

Using HS-SPME and portable gas-chromatography: This technique combined standard gas-generating device, flow-through sampling chamber and permeation tubes, similar to above. Standard gases were generated using NIST traceable permeation tubes, placed in a glass permeation tube holder and swept with a constant flow of dilution air. Wide range of concentrations for benzene, toluene and ethylbenzene were obtained by adjusting both airflow rate and permeation tube incubating temperature and successively quantified (Jia *et al*., 2000).

Liquid samples

Using headspace vials of different volumes: For determination of pthalate esters and triclosan in environmental waters, aliquots of 10 mL samples were placed in 22 mL headspace vials, Al cap with PTFE-faced silicone-rubber septum was used, vials were immersed in water bath and equilibrated for 5 min before analysis; DI-SPME was performed with the fiber immersed into the sample for 5-80 min with magnetic stirring (Polo *et al*., 2005). Calibration curves for phthalate esters were constructed under similar experimental conditions. SPME fiber was exposed to the sample for 30 min at room temperature (20°C). HS-SPME extractions were performed at room temperature and at 100°C whereas DI-SPME was performed at 20°C for generation of calibration curves for the analytes of interest for quantification (Canosa *et al*., 2005). Method of analysis for phenolic flame retardants in water samples was similar wherein 22 mL headspace vials were used for both HS-SPME and DI-SPME (Polo *et al*., 2006). For quantification of eucalyptol, camphor and borneol in chrysanthemum flowers, aqueous extracts of the same were subjected to SPME in 8 mL headspace vials at 70°C for 15 min under magnetic stirring conditions (Dong *et al*., 2007).

Using reduced pressure technique in combination with HS-SPME: At higher temperatures of extraction, handling of vials is difficult and pressure build-up inside the same can cause losses of sample vapor while removing the SPME needle. Hence, a combination of HS-SPME with reduced pressure was proposed to enhance extraction of analytes from the aqueous phase to the gaseous phase. Geometry of modified conical flasks were designed to allow a reduced headspace volume around the fiber while keeping the headspace to aqueous phase volume ratio constant and to allow a larger exchange surface between headspace and sample to improve analyte transfer from aqueous to headspace phase. A glass tube was welded at the neck of the flask in order to carry out HS-SPME in reduced pressure conditions. In case of HS-SPME sampling at atmospheric pressure, this opening was tightly shut. Both magnetic and mechanical stirring were tested. Calibration curves were constructed by this technique (combining the effects of pressure and agitation) for butyl and phenyltin compounds (Darrouzes *et al*., 2005). In environmental samples, the concentration of VOCs calibrated ranged widely from 10^{-12} ppt to 10 ppm.

From food samples: For quantitative analysis of VOCs in orange beverages, 5 g of diluted orange beverage emulsion (1:100) was transferred into a 20 mL vial containing a microstirring bar. The 1.5 g of NaCl and 1 μL of butyl acetate as internal standard was added to the vial. The vial was sealed with a teflon-lined septa and screw cap and immersed in water bath at 45°C. CAR/PDMS SPME fiber was exposed for 15 min at 45°C to reach equilibrium under stirring condition. The 5, 10, 15, 20 and 25 min were investigated to study equilibrium effect. Stock standard solutions of 20 mg L^{-1} (w/v %) of flavor compounds were individually prepared in deionized water. For quantitative analysis, the stock solution of myrcene and limonene were diluted to yield suitable concentration ranges of 4-40 and 30-300 mg L^{-1} (w/v %), respectively. Butyl acetate was used as an internal standard during quantification (Mirhosseini *et al*., 2007).

Similar reports are available for quantitative estimation of volatiles in wine solutions. About 7.5 mL aliquots of wine model solutions were transferred to 20 mL vials, corresponding to a liquid phase/headspace

volume ratio of 0.4. After the addition of 1.5 g of NaCl (0.2 g mL^{-1}), the vials were sealed with 8 mm PTFE/Silicone septum and Al caps and placed in a Combipal SPME automatic sampler with incubation oven. A single SPME fiber was inserted into each vial for 45 min at constant temperature (30, 40 and 50°C) with stirring at 500 rpm (Neto *et al*., 2007).

Based on the above discussed methodologies for detection of VOCs, experiments were designed to arrive at a simplistic laboratory set up for quantification of a few known VOCs for Salmonella contaminated meat samples.

MATERIALS AND METHODS

Researchers have conducted investigations on headspace gases associated with Salmonella contamination of sterile beef samples. Sterile beef (fresh strip loins) samples were inoculated with *Salmonella typhimurium* and both control and inoculated samples were stored at 20°C in 20 mL headspace vials, covered with food grade cling film. Acetic acid and ethanol were among the most promising compounds detected in the study using HS-SPME/GC-MS and these compounds could successfully be used in monitoring the status of Salmonella in raw fresh beef (Bhattacharjee *et al*., 2010). The similar studies on vacuum packaged beef strip loins (fresh and aged), repackaged on polystyrene trays and over-wrapped with food grade cling film established acetic acid to be an important headspace compound indicating Salmonella contamination (Bhattacharjee *et al*., 2011). From these findings, acetic acid and ethanol, being simple molecules to quantify, researchers proceeded for investigation of methods to quantify these as headspace gases.

Standardization of calibration procedure for meat volatiles: This involved development of calibration equations for acetic acid and ethanol to determine the range of concentration of these specific indicator compounds. Researchers have adopted two different approaches based on the generation methods of VOCs.

Vapor injection: Several dilutions of acetic acid vapors were prepared using Hamilton gas-tight syringes from acetic acid standard reagent (99.7% purity) stored in a 250 mL glass bottle. The vapors were injected into sampling gas chambers (headspace vials and flasks) and HS-SPME was carried out under static and dynamic conditions (magnetic stirring). The exact concentration of acetic acid vapors withdrawn into the Hamilton syringe and that injected into the sampling chambers were precisely calculated taking into account the substance partial pressure and actual needle volume (considering dead volume). A series dilution concentration calculator chart for each VOC was individually constructed in MS-Excel for this purpose. This chart was made use of in preparing series dilution of vapors of acetic acid used for calibration work (Table 1).

Preliminary trials of different concentrations of acetic acid at different SPME extraction time using 22 mL headspace vials showed that lesser extraction time (sec to very few minutes) was required for lower concentrations and higher extraction time (10-15 min) for higher concentrations.

However, to have a uniform protocol for generation of standard curves for acetic acid, 20 min extraction time was kept constant for all concentrations of acetic acid investigated. The vapors in the vial were allowed to equilibrate under static conditions for several minutes prior to extraction by SPME fiber for 20 min.

Under static conditions: The 22 mL headspace vials were used for generating calibration curve for acetic acid under static conditions using HS-SPME.

Use of 22 mL headspace vials

With Al crimp seals for vials: The 22 mL headspace vials were used to generate standard known concentration of acetic acid for a standard calibration curve. The vials were sealed with open center Al crimp seals lined with PTFE/silicone. GC/MS analysis of the SPME extract was performed in accordance to the method optimized for detecting the VOCs from the meat samples. However, no

Table 1: Series dilution concentration calculator of acetic acid

			Basic setup					
Variables	Values	Variables	Base C1 (sat.)[a]	N1[b]	C2[a]	N2[b]	C3[a]	N3[b]
Temperature (K)	298	Used volume of stock bottle of acetic acid (mL)	250	1	22	1	22	1
Atmospheric pressure (kPa)	101	Air (mol)	0.010191429	4.10388×10^{-5}	0.00094	4.29042×10^{-5}	0.00094	4.29891×10^{-5}
Substance partial pressure (kPa)	2.114	Acetic acid (mol)	0.000213314	8.53255×10^{-7}	8.53255×10^{-7}	3.87843×10^{-8}	3.87843×10^{-8}	1.76292×10^{-9}
Needle (1 mL) dead volume (mL)	0.0067	Mole fraction of acetic acid	0.020501581	0.02037	0.00091	0.00090	4.12692×10^{-5}	$4.10071\times1^{-0^{5}}$
R-Universal gas constant (J/mol*K)	8.314	ppm	20501.58077	20367.91	908.94	903.16	41.27	41.01

[a]Concentration of acetic acid withdrawn into Hamilton syringe; [b]Concentration of acetic acid injected into sampling vials

consistency and reproducibility of peak area values were obtained for the different concentrations experimented with acetic acid.

It was opined that the crimp on seals were not suitable for injections with Hamilton syringe (used for preparing series dilutions) and there could have been possible leakage of vapors from the vials. It was decided to change vial caps for further trials.

With polypropylene caps for vials: The 20 mm polypropylene hole caps with PTFE-silicone septa were used in these trials with similar experimental conditions as described above. However, no trend in peak area with concentration of acetic acid was observed. Since, agitation strongly influences HS-SPME extractions, it was opined that stirring would have to be introduced to improve the results (Martos and Pawliszyn, 1997).

Under dynamic conditions: Headspace vials (22 and 4 mL) and flasks were used for generating calibration curve for acetic acid under dynamic stirring conditions using HS-SPME.

Use of 22 mL headspace vials: The 22 mL headspace vials were sealed with 20 mm polypropylene hole caps with PTFE-silicone septa. Several dilutions of acetic acid were prepared as per the series dilution chart, described above. Each concentration prepared was allowed to equilibrate under static conditions at room temperature (~23°C) for 5 min and then under magnetic stirring conditions for 10 min in between successive dilutions. SPME was carried out for 20 min in stirring mode and then GC/MS analysis was performed. With these modifications too, the peak areas were all in the same order of magnitude. It was also, observed that with longer static equilibration time (up to 2 h); different peak area responses were obtained for the same concentration. It was difficult to ascertain the equilibrium time for each concentration in the 22 mL vials. Therefore, 4 mL headspace vials were experimented with in the successive trials.

Use of 4 mL headspace vials: Similar procedure as outlined in above section was carried out in 4 mL headspace vials in the concentration range of 1.49×10^{-7} to 509 ppm for acetic acid. A linear correlation (log-log plot) between peak area responses and concentrations of acetic acid in ppm were observed with coefficient of correlation, r = 0.999. However, this was observed with only three experimental points wherein log of concentration values were either zero or positive. The correlation between peak area and corresponding concentration for the entire range worked out was not reliable and inconsistencies in peak area responses were observed using this Calibration Method. There are reports on deviations observed using small headspace volumes due to formation of vortex of air bubbles inside the body of the vial under stirring conditions in aqueous media (Bocchini *et al*., 1999).

In the present case, although gaseous sampling was done, the conditions inside the vial can be fairly assumed to be similar to the conditions created in the aqueous media. Therefore, it is probable that the fiber would not always remain completely immersed in the sample-air mixture vapors and thus peak area responses were not uniform. Moreover, displacement effects of the adsorbed compound onto the fiber coating due to air vortex could have been pronounced. Thus, deviations were observed between replicates of the same concentration and a good linear trend of peak area response with concentration was not obtained. Alternate approaches were therefore, attempted as described in this study.

Use of 500 and 1000 mL 3-neck flasks: A 500 mL 3-neck flask was used for the trial. The three necks of the flask were sealed by polypropylene caps lined with PTFE-silicone septa; a thermometer was introduced through one neck of the flask; silicone tubings from an NMP05 micro-diaphragm pump and the SPME fiber were inserted through the other two necks. The actual working volume of the flask (the volume the vapors would occupy), the volume of the pump tubings and the time for which the pumps would have to run for mixing were determined. Acetic acid vapors were injected using Hamilton gas-tight syringes from acetic acid standard reagent as described in this study. Following injection of acetic acid vapor, the pump was switched on for a definite time period to ensure uniform mixing of the vapors with the air in the flask following which the SPME fiber was inserted into the flask for 20 min. The flask and the caps were flushed with nitrogen in between each concentration. However, all concentrations provided peak areas in the same order of magnitude. This was attributed to the fact that there were residual vapors of acetic acid in the pump tubings which intermixed with the vapors of acetic acid of the successive dilution and therefore peak area responses were similar for all the concentrations. It was not feasible to flush the pump set along with the tubings with nitrogen and thus this system could not be employed for further trials. At this point, it was also decided to change the septa to PTFE/ red rubber septa to prevent possible leakage during multiple SPME injections.

Liquid injection: To redress the limitations in the above trials, it was decided to evaluate the performance of

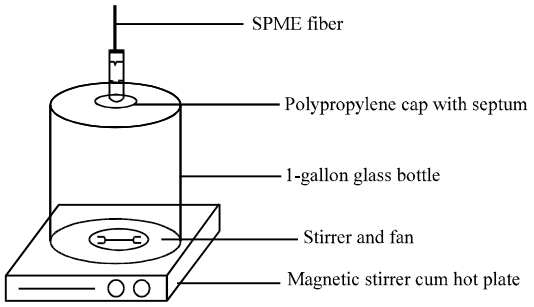

Fig. 1: Solid Phase Microextraction Extraction (SPME) System used in generating calibration curve in the laboratory

calibration using a 1 gallon container and generating vapors therein using direct liquid injection of the standard reagent or solution of the same prepared in an appropriate diluting reagent. Calibration was performed under dynamic mixing conditions.

A 1 gallon glass bottle with good mixing conditions (magnetic stirring and a fan-type circulator) was used to generate calibration curve for the VOCs (Fig. 1). The exact working volume of the bottle was determined. A circulating fan was placed at the bottom fitted with a cross-shaped magnetic stirrer. The circulating fan was wrapped with teflon tape to reduce adsorption of analytes on bottle wall and to prevent settling of organic dense vapors as reported by Bartelt (1997). A teflon injection port at the flask head was provided with a polypropylene cap lined with PTFE-silicone septum. Pure liquid standard was injected into the flask through the injection port using a 0.5-1 μL micropipette and successive dilutions of the same were prepared in methanol (spectroscopic grade) and injected by the same procedure. Methanol has been reported as a standard solvent of dilution for quantification using SPME (Canosa *et al*., 2005). Ethyl acetate and acetone (Tombesi *et al*., 2004) have also been reported as diluting solvents but were not used in the work since, these VOCs have been detected in the headspace of meat samples and successive work involving quantification of the same using these solvents as diluents would be inappropriate. A known concentration of the analyte vapors (C_{ppm}) was generated inside the gas chamber (flask) by direct liquid injection in accordance to the equation described by Nakamoto *et al*. (2006):

$$C_{ppm} = (\rho \times V_{vol} \times R \times T) \times 10^6/(M \times P_o \times V_o)$$

Where:

ρ = The density (g mL^{-1})
V_{vol} = The volume of analyte (liquid) injected (μL)
R = The universal gas constant (L atm)/(°K mol)
T = The temperature in Kelvin (K)
M = The molecular weight of the analyte (g/mol)
P_O = The pressure after vaporization of the VOC in the flask (atm)
V_O = The volume of the flask (mL)

The bottle was placed on a stirrer-hot plate and the liquid analyte or its dilution was injected through the septum provided in the cap. The flask was heated to 40°C and the vapors inside were magnetically stirred for 5 min to ensure uniform mixing of the analyte vapors with the nitrogen-air mixture inside the chamber. SPME fiber was then introduced through the sampling port and exposed to the gas chamber for 20 min under stirring conditions at 40°C. Post extraction, the SPME fiber was immediately desorbed in the GC/MS port for analysis. The flask along with the cap and injection port was flushed by purging ultrapure grade nitrogen gas in between successive injections. Three replicates for each concentration was performed. A fresh septum was used in each injection. This approach was evaluated for both acetic acid and ethanol.

Statistical analysis on the replication of the procedure: For construction of standard curves of acetic acid and ethanol, successive dilutions of the same were prepared and injected. The replication of the procedure was analyzed statistically and all statistical tests were conducted using STATISTICA 8.0 Software (Statsoft, OK, USA). For both acetic acid and ethanol, each dilution was injected in triplicate (i.e., three replicates) and three sets of peak areas were obtained for a particular concentration. One-way ANOVA studies were performed to analyze the replication procedure by comparing the mean peak areas of the three sets. A p-value of 0.05 was used to verify the significance of the tests.

RESULTS AND DISCUSSION

Using the Liquid Injection Method, calibration curves were generated for acetic acid and ethanol.

Standard curve for acetic acid: Pure liquid acetic acid (99.7%) was injected into the flask through the injection port using a 0.5-1 μL micropipette and successive dilutions of acetic acid were prepared in methanol (spectroscopic grade) and injected by the same procedure and the vapors analyzed using SPME-GC/MS.

The range of peak area obtained for acetic acid in the meat experiments was 10^5-10^8 and it was possible to obtain good linearity for acetic acid in the specified range.

Table 2: Concentration and peak areas of acetic acid (ppm) and ethanol (ppm) as obtained by HS-SPME /GC-MS analysis

Concentration of acetic acid vapor (ppm)	Peak area	Mean peak area	SD[a]	RSD[b] (%)
2E-10	4.6×10^5, 5.4×10^5	4.8×10^5, 4.9×10^5	42699.58	8.61
2E-8	7.6×10^5, 7.0×10^5	8.0×10^5, 7.5×10^5	50611.63	6.71
0.2	2.9×10^6, 2.9×10^6	2.9×10^6, 2.9×10^6	26978.47	0.92
1.0	1.2×10^7, 1.2×10^7	1.1×10^7, 1.2×10^7	875616.09	7.09
50	3.0×10^8, 3.4×10^8	3.4×10^8, 3.2×10^8	21310930.00	6.48
Ethanol vapor (ppm)				
0.02	1.2×10^4, 1.2×10^4	1.0×10^4, 1.1×10^4	1336.90	11.37
0.2	1.1×10^5, 1.1×10^5	1.1×10^5, 1.1×10^5	1958.74	1.68
1.0	1.3×10^6, 1.3×10^6	1.2×10^6, 1.2×10^6	86628.48	6.67
50	3.0×10^7, 3.1×10^7	3.3×10^7, 3.1×10^7	1541117.73	4.85
550	5.3×10^7, 5.6×10^7	5.4×10^7, 5.4×10^7	1328488.91	2.42

[a]Standard deviation; [b]Relative standard deviation; SPME = Solid Phase Microextraction

Table 2 gives the concentration and peak area data of acetic acid and the corresponding Relative Standard Deviations (RSD %) for replicates (three) of each concentration. A linear regression equation which denotes the mean peak area of acetic acid as a function of its concentration (ppm) is provided:

$$Y = 1989373 + 6494788 \times \text{Concentration} \quad (4)$$

where, Y is the mean peak area of acetic acid as obtained by HS-SPME/GC-MS analysis. The regression coefficient R obtained using this equation is 0.99 which indicates very good linearity in the concentration range of 2E-10-1 ppm corresponding to peak areas of 10^5-10^8.

Standard curve for ethanol: Similar procedure as above was adopted to generate standard curve for ethanol using the 1 gallon chamber. The range of peak area obtained for ethanol in the meat experiments was 10^4-10^7 and it was possible to obtain fairly good linearity for ethanol in the specified range. Table 2 gives the concentration and peak area data of ethanol and the corresponding Relative Standard Deviations (RSD %) for replicates (three) of each concentration. Figure 2 gives log-log plot of the standard curve for ethanol. The linear regression equation which denotes the mean peak area of ethanol as a function of its concentration (ppm) is provided:

$$Y = 6568422 + 90171 \times \text{Concentration} \quad (5)$$

where, Y is the mean peak area of acetic acid as obtained by HS-SPME/GC-MS analysis. The regression coefficient R obtained using this equation is 0.88. Similarly the log of mean peak area of ethanol can be obtained as a function of log of its concentration by the equation:

$$Y = 6.1974 + 0.6175 \times \text{Log}_{10}(\text{Concentration}) \quad (6)$$

where, Y is the Log_{10} (Mean peak area of acetic acid). The regression coefficient R obtained using this equation is 0.97. Therefore from Eq. 5 and 6, it could be concluded

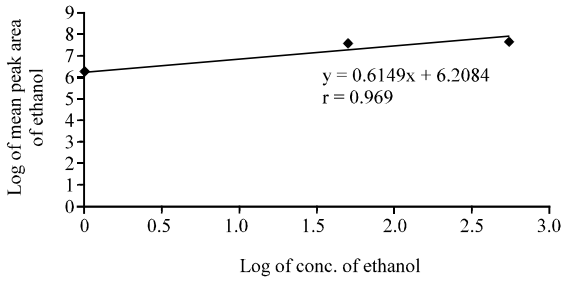

Fig. 2: Log-log plot of concentration and mean peak area of ethanol as obtained by HS-SPME/GC-MS analysis

that a good linearity was not obtained in the concentration range of 0.02-550 ppm corresponding to peak areas of 10^4-10^7 as shown in Table 2 and Eq. 5 while the log-log concentration curve Fig. 2 and Eq. 6 showed better linearity.

A better correlation coefficient was obtained for acetic acid compared to ethanol which can be attributed to the fact that ethanol being less volatile than acetic acid, losses of the same could have possibly occurred from the 1 gallon system at 40°C while performing SPME extractions.

Statistical analysis on the replication of the procedure: From one-way ANOVA studies, p-values of 0.97 and 0.98 were obtained for acetic acid and ethanol, respectively. Therefore, it can be reasonably concluded that there is insignificant differences in the peak areas of the three sets of acetic acid and ethanol and hence the replication procedure is statistically significant at 5% level of significance.

CONCLUSION

The comprehensive review on detection of VOCs in food and environmental samples enables us to design a simple laboratory model well suited for calibration of simple VOCs, individually without use of any elaborate set ups such as standard gas generators, mass flow

controllers and permeation tube assemblies. Good reproducibility was obtained with acceptable RSDs for both acetic acid and ethanol and linear correlations obtained for their concentration curves were also appreciably good.

The challenge in generating calibration curves for single gaseous analytes is to ensure an exact concentration of the analyte vapor to be created inside the gas chamber and to ensure uniform mixing of the sample vapors with air/nitrogen. Modifications in the calibration curves above are expected if the VOCs are combined for determination by HS-SPME/GC-MS. In presence of more than one VOC, construction of calibration curve will have to take into account inter-analyte displacement effects and competitive adsorption in the fiber coating (Augusto *et al*., 2001). This would make quantification particularly difficult. Further research needs to be done to quantify these VOCs in combination. Different statistical analyses such as regression equations and ANOVA conducted on the experimental data concluded that the mean peak areas of acetic acid and ethanol could be satisfactorily correlated linearly with their respective concentrations and that the replication studies are statistically significant at 5% level of significance. Real time sample analyses using this approach are suggested.

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