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Analysis of Pyrazine and Volatile Compounds in Cocoa Beans Using Solid Phase Microextraction

Analisis Pirazin dan Senyawa Volatil pada Biji Kakao Menggunakan Mikroekstraksi Fase Padat

Misnawi[1*)] and Ariza Budi Tunjung Sari [1)]

Summary

Analysis of pyrazine and volatile compounds in cocoa beans was done by using solid phase microextraction (SPME), to develop efficient non solvent extraction method. Extraction was carried out in head space technique using stableflex fiber coated with DVB/Carboxen/PDMS applied on manual sampling SPME Holder. Five grams of roasted fermented cocoa bean was processed into butter and placed into 30 *ml* vial and capped with a rubber septum, then heated at temperature of 70^{O}C for 30 min for the extraction. The fiber then was placed in GC header for desorption and separation. Results of the study showed that the SPME extracted pyrazines were adequate and well detected in a gas chromatography system. Peak area resulted from SPME covered 2.83–5.35% peak area from syringe, however SPME had comparable ability to syringe in extracting volatile compounds. Five most common pyrazines in cocoa bean aroma were identified, such as 2 methyl pyrazine (2MP); 2.3 and 2.5 dimethyl pyrazine (DMP); and 2,3,5 trimethyl pyrazine (TrMP) and tetramethylpyrazine (TMP). Other corresponding compounds were also detected in cocoa liquor, i.e. alcohols, carboxylic acids, aldehydes, ketons, esters, pyrazines, amines and other volatile compounds and strongly associated to chocolate aroma. The successful extraction of pyrazine and volatile-semi volatile compounds which contribute to chocolate aroma indicates SPME is applicable in flavor analysis.

Key words: Cocoa bean, flavour, pyrazine, solid-phase microextraction, head space, extraction, gas chromatography, maillard.

Ringkasan

Analisis pirazin dan senyawa volatil pada biji kakao dilakukan dengan perangkat mikroekstraksi fase padat (solid phase micro extraction, SPME)*, untuk mengembangkan metode ekstraksi tanpa pelarut yang efisien. Perangkat SPME dilengkapi fiber stableflex dengan polimer DVB/Carboxen/PDMS yang menjerap senyawa volatil di area* headspace*. Biji kakao terfermentasi disangrai dan diambil lemaknya untuk ditempatkan dalam botol bertutup septa. Sampel dipanaskan pada suhu 70^{O}C dan serat SPME ditusukkan menembus septa untuk mengekstrak senyawa volatil dari lemak kakao selama 30 menit. Senyawa volatil lemak kakao akan dijerap oleh serat SPME dan dilepaskan kembali untuk analisis kromatografi gas. Penelitian menunjukkan pirazin dan senyawa volatil yang diekstrak oleh serat SPME dapat terdeteksi dengan baik oleh kromatografi gas. Area puncak yang dihasilkan SPME meliputi 2,83–5,35% dari area puncak yang dihasilkan*

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syringe*, kendati demikian kemampuan ekstraksi SPME dapat disetarakan dengan* syringe*. Lima jenis pirazin yang sering terdapat di biji kakao telah diidentifikasi, meliputi metil pirazin (2MP); 2,3 dan 2,5-dimetilpirazin (DMP); dan 2,3,5 trimetilpirazin (TrMP) dan tetrametil pirazin (TMP). Senyawa lainnya juga terdeteksi meliputi alkohol, asam karboksilat, aldehida, keton, ester, pirazin, amin dan senyawa volatil lainnya, dan diketahui erat kaitannya dengan aroma khas cokelat. Keberhasilan SPME dalam ekstraksi pirazin dan senyawa volatil-semi volatil yang berperan penting dalam pembentukan aroma cokelat menandakan SPME dapat digunakan lebih lanjut untuk analisis citarasa.*

INTRODUCTION

Pyrazines (1,4 diazines) in roasted cocoa bean contribute to a pleasant aroma of chocolate, and associate with good quality of roasted cocoa bean perception. Large number of pyrazines have been identified in cocoa and are considered extremely important in cocoa flavor (Brunetto *et al.,* 2009; Frauendorfer & Schieberle, 2008; Krings *et al.*, 2006; Bonvehi & Coll, 2002; Counet *et al*., 2002; Jinap *et al.*, 1998). Detection of volatile compounds including pyrazine has been developed using gas chromatography which requires extraction of those compounds from solid or liquid sample. Schultz *et al*. (1977) developed a method of extraction using simultaneous distillation and extraction (SDE), in which sample is water-steamed resulting evaporation of volatile compounds which then being captured by certain solution and concentrated prior to syringe injection into GC system. This SDE-syringe method involves high temperature to extract and move the volatile compound into trapping solution which could detriment the nature of pyrazine. Since pyrazines are product of heating process, bias in analysis could be occurred due to uses of high temperature during steam distillation.

Solid phase microextraction (SPME) is developed to allow extraction of volatile compounds without use of any solvent. SPME is a solventless extraction technique introduced by Berlardi & Pawliszyn (1989), which binds volatiles on a silica fiber coated by polymer in headspace area of sample (Berlardi & Pawliszyn, 1989). Exposing fiber at headspace area ensures that only volatile compounds reach and contact to fiber. Extractions can be conducted in room temperature (Vazquez-Landaverde *et al.,* 2008; Marsili, 2002), but moderate heat often increase volatility (Ducki *et al.*, 2008). Trapped volatile then was released in the GC system during injection through desorption mechanism (Pawliszyn *et al.,* 1997).

SPME differs due to its type of fibers; polar, non-polar and bipolar fibers. Each type of fiber adsorbs different type of compounds. Polar fiber may be coated by polyacrylate (PA) (Shirey, 1999) or carbowax-divinylbenzene (CW-DVB) (Shirey & Sidisky, 1999). Nonpolar fiber may be constructed of polydimethyl siloxane (PDMS). While bipolar fibers are combination between polar and nonpolar polymer. Bipolar fiber could capture larger spectrum of compounds and could be developed from materials of polydimethylsiloxane-divinylbenzene (PDMS-DVB) or Carboxen-PDMS (Shirey & Sidisky, 1999).

This research investigated analysis of pyrazine and volatile compounds of cocoa beans extracted by SPME and evaluated toward SDE-syringe extraction. Analysis was brought by gas chromatography-mass spectrometry, focusing on pyrazine analysis especially 2-methylpyrazine (2-MP);

2,5-dimethylpyrazine (2,5DMP); 2,3-dimethylpyrazine (2,3-DMP); 2,3,5-trimethylpyrazine (TrMP) and tetramethylpyrazine (TMP).

MATERIAL AND METHODS

Cocoa beans used were fermented bulk cocoa beans obtained from Post Harvest Laboratory of Indonesian Coffee and Cocoa Research Institute (ICCRI). The beans were roasted at temperature of 150^{O}C for 45 min and manually processed for shell removal. A quantity of roasted beans was pressed to collect the butter for pyrazine analysis, while the other was ground to obtain cocoa liquor for volatile compounds analysis.

Gas chromatography system consisted of Shimadzu GC-2010 equipped with Rtx-1 (100% dimethyl polisiloxane) column and flame ionization detector for pyrazine analysis, and mass spectrometry for volatile compounds analysis. Bipolar fiber PDMS-DVB SPME from Supelco was used for extraction.

Method of extraction

Referring to successful pyrazine extraction from peanut butter (Supelco, 1998), this study used cocoa butter as the base of pyrazines extraction. Three 30 mL vials, each contained five grams of roasted fermented cocoa butter and capped with a rubber septum were immersed in the waterbath. To observe effect of heat to volatile compounds extraction, immersion was prepared at temperatures of 50^{O}C, 60^{O}C and 70^{O}C respectively. The vial was heated for 30 min for the extraction. The fiber was then placed in GC injector for desorption and separation in capillary column.

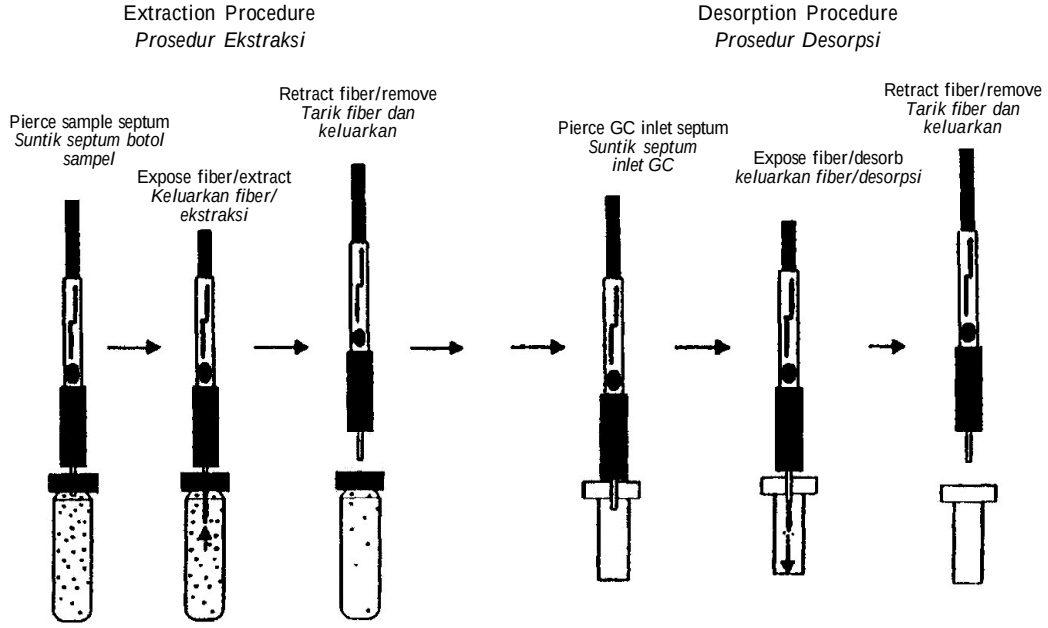

Figure 1. Mechanism of extraction and desorption of SPME.

Gambar 1. Mekanisme ekstraksi dan desorpsi SPME.

Table 1. GC conditions applied in Supelco (1998) and Misnawi *et al.* (2004) pyrazines detection and modified methods

Tabel 1. Parameter kromatografi gas berdasarkan metode Supelco (1998) dan Misnawi et al. (2004)

Properties *Parameter*	Supelco (1998)	Misnawi *et al.* (2004)	Modified
Sample preparation and injection *Penyiapan contoh dan injeksi*	Headspace SPME-SPME	Steam Destillation-SPME	Headspace SPME-SPME
Injector Parameter *(Parameter infektor)*			
Inlet Liner	0.75 mm	4 mm	0.75 mm
Temperature *(Suhu)*	270°C	200°C	splitless/260°C
Pyrazine source *(Sumber pirazin)*	Peanut	Cocoa bean	Cocoa butter
Column *(Kolom)*	Supelcowax 1030 m x 0.25 mm ID x 0.25 μm	HP-20 M50 m x 0.32 mm ID x 0.3 μm	Rtx-1(Dimethyl Polysiloxane) 30 m x I,25 mm ID x 0.25 μm
Oven *(Oven)*			
Initial temperature *(Suhu awal)*	40°C	60°C	60°C
Equilibrium *(Keseimbangan)*	5 min	3 min	3 min
Rate *(Laju)*	4°C/min	5°C/min	5°C/min
Final temperature *(Suhu akhir)*	230°C	180°C	200°C
Hold	-	5 min	-
Detector *(Detektor)*	Ion trap mass spectrometer, selected ions used for quantification Spectrometry	Flame Ionization Detector (FID)	Flame Ionization Detector (FID) for pyrazine analysis, Mass (Shimadzu GC-MS 2010)

GC Conditioning

Gas chromatography adjustment used in this study was obtained from several preliminary experiments to obtain the best response of the GC system on pyrazine compounds separation and detection. Two methods were used as references i.e.a method for peanut pyrazine detection with SPME extraction from Supelco (1998) and GC condition for cocoa pyrazine detection with SDE (simultaneous distillation extraction) developed by Misnawi *et al.* (2004). Those methods were combined to obtain a new detection method as described in Table 1.

RESULTS AND DISCUSSION

Performance of SPME

The bipolar fiber PDMS-DVB used in this experiment was able to extract pyrazine from cocoa butter and release volatile compounds for further analysis in GC. Supelco (1998) recommends using polar fiber for optimum extraction of polar compounds and vice versa. Bipolar fiber is expected to provide larger spectrum of adsorption than the other two types of fiber. PDMS-DVB bipolar fiber was employed to obtain most of pyrazine in cocoa butter.

The extraction of both pyrazine and volatile compounds in fiber was through adsorption mechanism. There are two possibilities of how compounds attached on the fiber, by absorption or adsorption. Absorption mechanism is when compounds bind chemically with the matrix, while in adsorption mechanism, compounds only adhere on the surface of matrix. After being adsorbed onto fiber surface, volatile compounds subsequently were released by certain desorption procedure.

Extraction of analytes will continue running until the fiber reach its equilibrium state. At equilibrium point, concen-

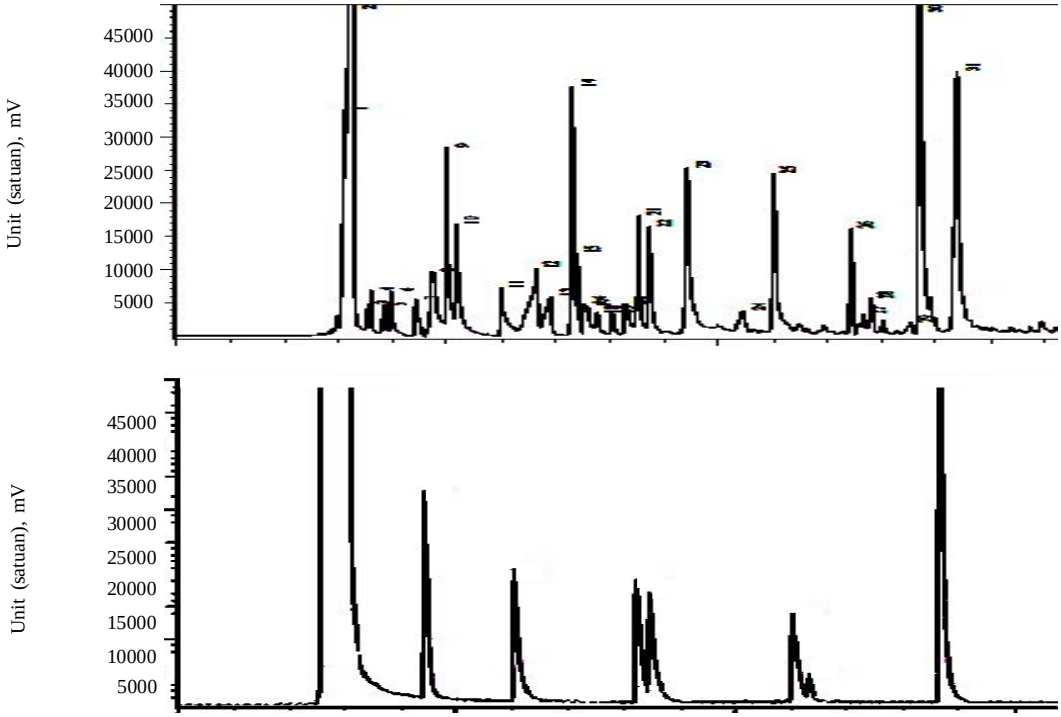

Figure 2. Chromatogram of volatile compounds extracted from cocoa butter by using SPME (above) and seven pyrazine standards (below).

Gambar 2. Kromatogram senyawa volatil yang diekstrak dari lemak kakao menggunakan SPME (atas) dan senyawa pirazin yang dijadikan standar (bawah).

tration of analyte in the fiber is equal with concentration of analyte in sampling area, Further exposion will result in minor effect, since the fiber would meet its limit of trapping. Equilibrium state usually accomplished in 30 minutes (da Silva, 2008; Marsili, 2002).

The extraction process was followed by injection to GC instrument. There are two methods of setting GC parameters, the first developed by Supelco and the second developed by Misnawi *et al.* (2004). Supelco has successfully analyzed pyrazines from peanut butter, while Misnawi *et al.* (2004) have analysed pyrazine from cocoa bean by applying SDE extraction.

Adjustment made to those methods, injector temperature was referred to Supelco methods that apply injector temperature of 230^{O}C. Injector temperature above 200^{O}C was recommended to accommodate desorption of analyte from fiber. This is also confirmed by works of Kumazawa *et al.* (1999), Vilchez *et al.* (2001) and Perraudine *et al.* (2006) that applied injector temperature over than 200^{O}C. Therefore column constructed of *dimethylpolisiloxane-crossbond* were applied due to its ability to retain high temperature up to 300^{O}C.

The modification allowed better component separation and took relatively shorter time. Chromatogram of the modified method in the volatile compounds separation is shown in Figure 2. Standard solution consisting of 2-methylpyrazine (2MP); 2,5 dimethylpyrazine (2,5 DMP); 2,3 dimethylpyrazine (2,3 DMP); 2,3,5 trimethylpyrazine and 2,3,5,6 tetra-

methylpyrazine (2,3,5,6 TMP) were extracted by SPME apparatus. Beside pyrazines, numbers of volatile and semi-volatile compounds also appeared, such as carboxilyc acid, hydrocarbons, alcohols, aldehides and esters.

This result showed that the developed method was able to detect most of pyrazine compound presented in roasted cocoa bean. The 2,5-DMP, 2.3-DMP, 2,3,5-TrMP and tetramethylpyrazine were detected at retention time of 8.539, 8.733, 11.088 and 13.715 minutes, respectively. Due to its molecular weight which affects volatility, 2MP was among the compounds identified in early minutes, along with simple carboxylic acids, aldehyde and alkane. Detection of 2MP was followed by 2,5 DMP; 2.3 DMP; 2,3,5 TMP and acetylpyrazine.

Compared to SDE-syringe application, concentration of extracted compounds using SPME was much smaller. Peak area resulted from SPME analysis covered 2.83–5.35% of peak area resulted from syringe (Figure 3). When sampling was carried out using syringe, all molecules in sample was inhaled into the syringe. While in extraction carried out using SPME, the molecules will be trapped in limited amount. Adsorbing rate of SPME decreased as the concentration of analyte increased (Figure 4). This occurred as the fiber became saturated and was unable to trap additional molecules. This saturation stage suggested equilibrium state that is normally accomplished in 30 minutes.

The molecules adsorbed in various quantity, depends on thickness of polymer as well as their own physical properties such as polarity and boiling point. As being shown in Figure 5, peak area resulted by syringe extraction shows that 2MP has larger peak area then 2,3 DMP > 2,5 DMP > 2,3,5 TrMP. While peak area resulted from SPME shows different sequence, where peak area of 2,3 DMP > 2,3,5 TrMP > 2 MP > 2,5 DMP.

The difference might be due to compound volatility, and also due to difference in polarity. Interaction between compounds and SPME polymer was suggested to occurr within polarity variations. Matrix effect has been an issue related to the use of SPME, which affects extraction-desorption performance of SPME fiber (Górecki *et al.,* 1999). However by specifying target compound and selecting suitable fiber, this matrix effect could be minimized.

Effect of extraction temperature

Extraction temperature was set at three levels, 50^{O}C, 60^{O}C and 70^{O}C. GC analysis detected 27 compounds, 33 compounds and 34 compounds, respectively for extraction temperature of 50^{O}C, 60^{O}C and 70^{O}C. Temperature of 70^{O}C facilitated liberation of volatile compounds. At this condition, cocoa butter as the matrix melts down and provides ways for volatile compounds to evaporate (Figure 6).

Extraction temperature also affected peak area performed by volatile compounds. Compounds of 3-methyl-butyl-acetate; 2,5-dimethyl pyrazine and 2,3-dimethyl-pyrazine were found in larger peak area at temperature 50^{O}C, and decreasing as the extraction temperature raised. However benzaldehyde, phenylethyl alcohol and 2-phenylethyl ester showed inclination in peak area at extraction temperature 60^{O}C and 70^{O}C. This result indicated that moderate heat facilitated more deliberation of compounds in high molecular weight.

Heat available in the extraction phase also supplies energy to molecules to reach its boiling point. 2-methyl pyrazine under 76 cmHg, has boiling point of 135^{O}C; 2,3-dimethyl pyrazine and 2,5-dimethyl

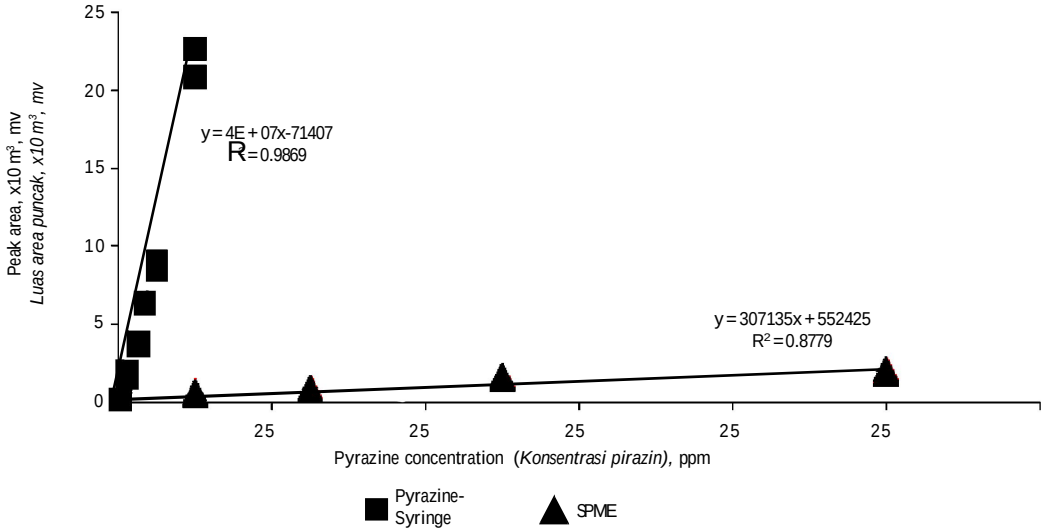

Figure 3. Comparison of peak area resulted from syringe extraction and SPME

Gambar 3. Perbandingan luas area puncak yang dihasilkan ekstraksi syringe dan SPME.

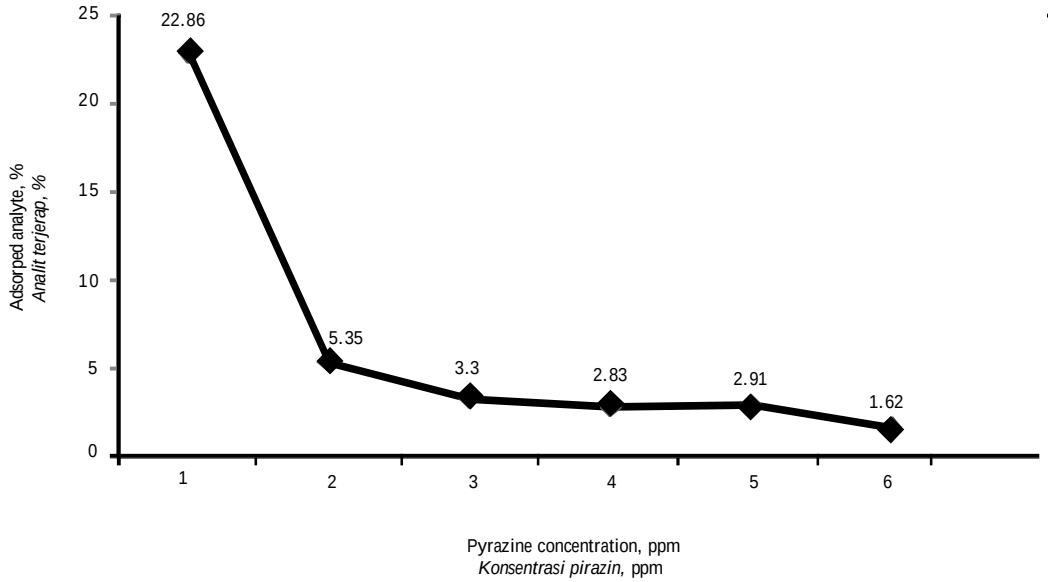

Figure 4. Effect of pyrazine concentration on the percentage of adsoped analyte.

Gambar 4. Pengaruh konsentrasi pirazin terhadap persentase analit terjerab.

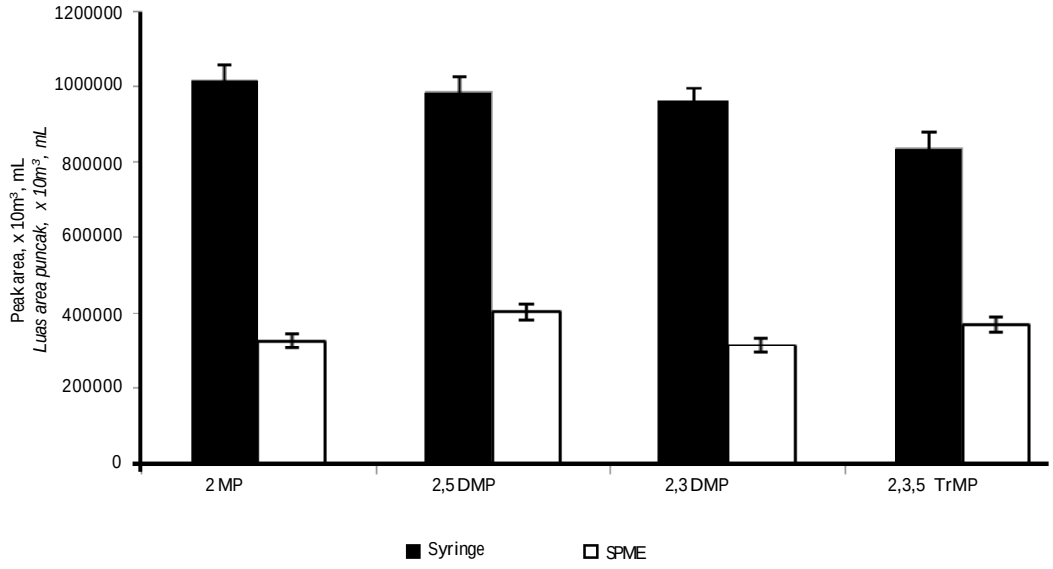

Figure 5. Comparison of peak area of four pyrazines extracted by syringe dan SPME.
Gambar5. Perbandingan luas area empat pirazin yang diekstrak dengan syringe dan SPME.

pyrazine have boiling point of 155^{O}C, while 2,3,5-trimethyl pyrazine has boiling point of 171^{O}C (Burdock, 2005).

Most of pyrazines underwent loss of quantity during heat treatment, as occurred in pyrazine, 2-MP, 2,5-DMP, 2,3-DMP and acetylpyrazine (Figure 7). Significant loss was found in 2,5-DMP and ACP, where the compounds were almost eliminated. Slight reductions were found in pyrazine and 2-MP, which the final amount was is somewhat less than it was.

Instead of heat loss, TMP and TrMP underwent increasing quantity at higher temperature. TrMP was suggested being synthesized during heat treatment, as being showed by gradual supplementation. Additional TMP was detected in 70^{O}C, after minor reduction in 60^{O}C.

Extraction of Cocoa Volatile Compounds by using SPME

Detection of other volatile compounds in cocoa bean extracted by SPME was performed using GC-MS. Complete separation of cocoa aroma chromatograms were obtained during running time for total 36 min, yet the chromatogram at 30 min running time showed small peak areas. Identification by using GC-MS library showed that the major peaks were acetic acid, tetramethyl pyrazine, 3-methyl pentanoic acid and 2,3-dimethyl oxirane exposed at retention time (RT) of 17.54, 18.26, 22.52 and 25.99 min, respectively. Other volatile compound such as dodecanoic acid (RT 26.12), benzene-acetaldehyde (RT 28.59) and 1,4-bis (morpholinoacetyl)piperazine (RT 32.70) were detected in small peak areas.

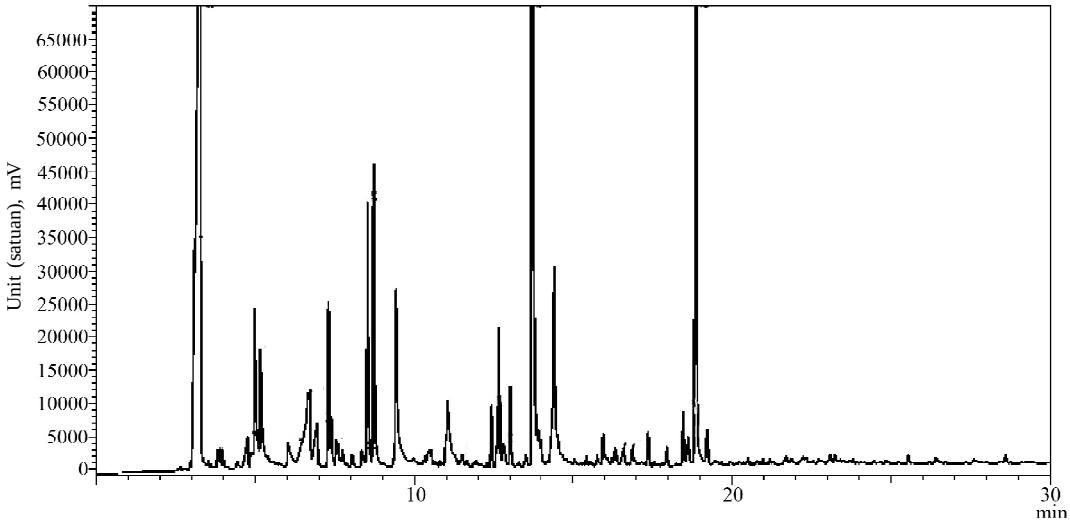

Figure 6. Chromatogram of cocoa butter, headspace extraction in 70°C.

Gambar 6. Kromatogram senyawa volatil lemak kakao, ekstraksi headspace pada 70°C .

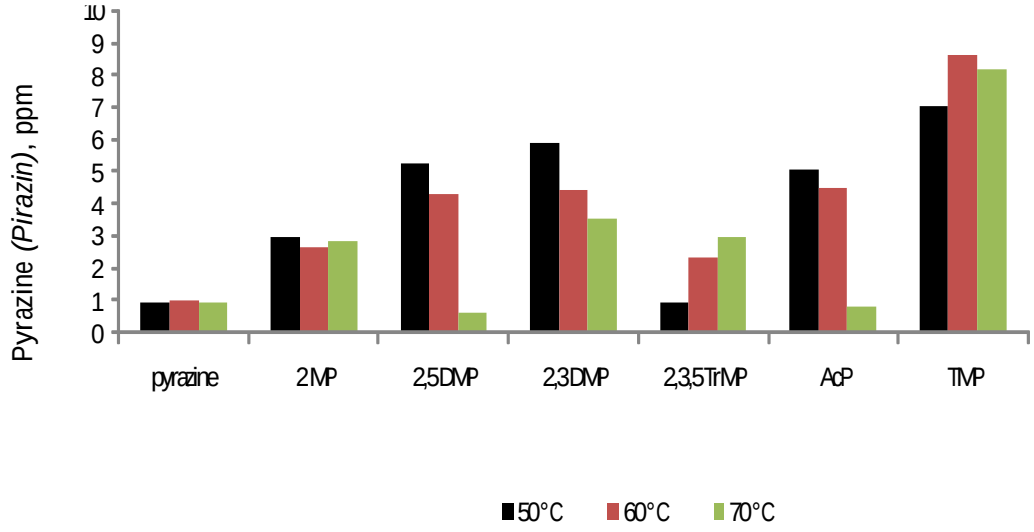

Figure 7. Effect of extraction temperature on seven pyrazine compounds.

Gambar 7. Pengaruh suhu ekstraksi terhadap konsentrasi 7 senyawa pirazin.

Analysis showed that 36 compounds were detected in fermented cocoa liquor volatile compounds which representing alcohols, carboxylic acids, aldehydes, ketons, esters, pyrazines, amines and other volatile compounds (Table 2). They were most volatile compounds associated in fermented cocoa aroma. Frauendorfer and Schieberle (2006) identified 35 most active compounds from cocoa powder extract off however Jinap *et al.* (1998) stated that the main aroma compounds contribute to chocolate

Table 2. Volatile compounds of cocoa beans extracted by SPME

Tabel 2. Senyawa volatile dari biji kakao yang diekstrak menggunakan SPME

Volatile compounds *Senyawa volatil*	Coresponding odour	Volatile compounds *Senyawa volatil*	Coresponding odour
Alcohol *(Alkohol)*		Ester	
2,3-Butanediol	sweet, creamy	1-Butanol, 3-methyl-, acetate	lemon-like, flowery, sweet, fruity
Phenylethyl Alcohol	caramel-like, alcohol-like, sweet	Acetic acid, 2-phenylethyl ester	cereal-like, roasted cocoa
Cyclobutanol	roasted cocoa	Propanol, methoxy-, acetate	flowery, green
2-Nonanol	no smell	Benzeneacetic acid, ethyl ester	nutty, bean-like
2-Heptanol	no smell	Decanoic acid, ethyl ester	nutty
1,6-Octadien-3-ol, 3,7-dimethyl-	bean-like	Dodecanoic acid, ethyl ester	sweet, creamy
Carboxylic acid		Propanol, methoxy-, acetate	no smell
Acetic acid	sour, nutty	Octanoic acid, ethyl ester	alcohol-like
Pentanoic acid, 3-methyl-	sweet, rancid	Pyrazines	
Octanoic Acid	sweet, chocolate-like	Pyrazine, trimethyl-	sweet,nutty, bean-like, smoky
n-Hexadecanoic acid	no smell	Pyrazine, tetramethyl-	bean-like, chocolate, rancid
Butanoic acid, 3-methyl-	sour	2,3-Dimethyl-5-ethylpyrazine	roasted cocoa
Aldehyde		2,3,5-Trimethyl-6-ethylpyrazine	no smell
Benzaldehyde	bean-like	Amines	
Benzeneacetaldehyde, alpha ethylidene-	caramel-like, smooky, nutty	1,4-Bis(morpholinoacetyl) piperazine	caramel-like, sweet
5-Methyl-2-phenyl-2-hexenal	roasted cocoa	7H-Pyrrolo(2,3-d)pyrimidin-4-amine	caramel-like
Keton		1,2-Propanediamine	citrus-like
Ethanone, 1-(1H-pyrrol-2-yl)-	sweet, caramel-like, honey- like, nutty	Other volatiles	
Cyclobutanone, 2-ethyl-	no smell	Oxirane, 2,3-dimethyl-, trans-	smoky
2-Butanone, 3-hydroxy-	no smell	Propane, 2-(ethenyloxy)-	caramel-like
2-Nonanone	no smell	Hydrazine, 1,1-dimethyl-	roasted cocoa

flavor are pyrazine, carbonyl, ester, alcohol, hydrocarbon and phenol.

This result also implies that cocoa bean aroma is characterized by presence of sweet, caramel-like, nutty and bean-like odour. Those odours are expressed by pyrazines, ethyl ester and alcoholic compounds, particularly trimethylpyrazine, tetramethylpyrazine, 2,3-butanediol, dodecanoic acid, phenylethyl alcohol, ethanone, benzeneacetal-dehyde and 1,4-bis (morpholinoacetyl) piperazine. Few unusual odours might also present, for instance rancid and lemon-like that came with 1-Butanol, 3-methyl-, acetate and 3-methyl-pentanoic acid.

CONCLUSION

SPME offers accurate, easier extraction technique, shorter extraction time and to extract specific compounds with lower extraction temperature. Selection can be made by choosing the type and fiber specification. SPME extracted pyrazine was adequate and well detected in a gas chromatography system equipped either with FID or Mass Spectrometry detector. Over thirty compounds were detected as the most representative volatile-semi volatile compounds from roasted cocoa beans including 2 methyl pyrazine (2MP); 2,3 and 2,5 di-methyl pyrazine (DMP); and 2,3,5 trimethyl

Pyrazine (TrMP) were identified. Alcohols, carboxylic acids, aldehydes, ketons, esters, pyrazines, amines and other volatile compounds were also extracted and associated to chocolate aroma. The presence of other volatile compounds which are the key contributor to chocolate aroma indicates SPME extraction is applicable in aroma analysis. Limiting factor for SPME is in quantity of compound trapped from the extraction which is lower than that of resulted from syringe injection.

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