Study on Supercritical Fluid Extraction of Aromatic Compound from Roasted Cocoa Beans Using Multilevel Factorial Design

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Abstract: Extraction of aromatic compound from cocoa beans was carried out using Supercritical Fluid Extraction (SFE) in order to obtain natural cocoa extract for perfume formulation. The experiment was carried out by multilevel factorial design to investigate which factors; pressure (100, 125, 150 & 200 bar) and time (30, 45, 60 and 75 minutes), influence the extraction condition of cocoa aromatic (5-methyl-2-phenyl-2-hexenal), fatty acid (hexadecanoic acid) and alkaloid (caffeine) compounds. It was found out that the factors influence, significantly, yield of extract and extraction of fatty acid compound, while only pressure significantly affected amount of aromatic compound extracted. Extraction with high amount of aromatic compound (24.5mg/g extract) was obtained at 200 bar and 45 minutes. This result is used as a based to investigate further on the optimum condition to obtain high yield of aromatic compound in order to formulate the natural cocoa-scented perfume.

Keywords: Roasted Cocoa Bean, Supercritical Fluid Extraction, Aromatic Compound

Introduction

Cocoa aroma is contributed by at least by eleven groups of compounds (Table1), including pyrazines, esters, carbonyls, phenols and hydrocarbons (Jinap, et al. 1998; Jinap, et al. 2004). Acidic compounds such as acetic, isobutyric, isovaleric, hexanoic and pentanoic acids were reduced during roasting (Krysiak, et al. 2007). Van Praag, et al. (1968) suggested that cocoa aroma was contributed by aldehydes and pyrazines by extraction using steam distillation in three portions (acidic, neutral and basic). The basic fraction consists of compounds with nutlike smelling components such as alkyl substituted pyrazines, while the acidic fraction gave the slight phenolic odor. Among these three fractions, the neutral fraction was the most important as it contains 5-methyl-2-phenyl-2-hexanal which possess deep and bitter persistent cocoa note together with isovaleraldehyde and methyl disulfide. Furthermore, Bonvehi, et al. (2005) identified that 5-methyl-2-phenyl-2-hexenal gave the intense bitter taste to cocoa, as well as 4-methyl-2-phenyl-2-pentenal. In addition, chocolate aroma comes from 2,3,5,6-tetramethylpyrazine, while cocoa and roasted nuts aroma comes from 2,5-dimethylpyrazine. Caramelizing of sucrose during roasting increases furans compound in roasted cocoa beans.

Table 1: Examples of compounds contributes to cocoa aroma in roasted cocoa beans

Group	*Compounds (examples)*	*Aroma attributes*	***References***
Pyrazines	2,3,5,6-tetramethylpyrazine 2,5-dimethylpyrazine	Chocolate, cocoa, coffee cocoa and roasted nuts	Jinap, et al (1998) Bonvehi, et al (2002), Ducki, et al (2008)
Esters	Butyl acetate Ethyl benzoate	Fruity taste and aroma Fatty, fruity	 Bonvehi, et al (2005)
Carbonyls; aldehydes	5-methyl-2-phenyl-2-hexanal 4-methyl-2-phenyl-2-pentanal	deep and bitter persistent cocoa note	Bonvehi, et al (2005)
Phenols	Vanillin (phenolic aldehyde)	vanilla	Bonhevi, et al (2005)
Alcohols	2- heptanol Linalool [3,7-dimethylocta-1,6-dien-3-ol] Phenyl ethyl alcohol [2-phenylethanol]	fruity, herbal, sharp flowery aromatic chocolate note	 Frauendorfer, et al (2008)
Monoterpene hydrocarbons	Pinene Dimethyl-octane	Lemon like	Ducki, et al (2008)
Alkaloids	Caffeine Theobromine	coffee	Ducki, et al (2008)
Furans	2-methylfuran, tetrahydro-2-methylfuran	Sweet and caramel-like aroma of burnt sugar	Krings, et al (2006) Frauendorfer, et al (2008)
Acids	Acetic acid, Valeric acid, Butyric acid, Hexanoic acid, Pentanoic acid	Vinegar	Krysiak, et al (2007) Krings, et al (2006), Ducki, et al (2008)

Voight, et al. (1994) reported that the cocoa-specific aroma precursors were derived from the fermented cocoa seeds. The characteristic aroma of cocoa is a result of crude fermentation of fresh seeds, followed by drying and roasting (Bixler, et al. 1999). During the fermentation, fresh cocoa beans go through complex transformations: (1) the sugars from the mucilaginous pulp of the seeds are rapidly metabolized, producing volatile and nonvolatile organic acids; (2) the degradation of proteins to form peptides and free amino acids; (3) oxidation of polyphenols to form insoluble compounds, mainly *o*-quinones; and (4) hydrolysis of glycosides (mainly anthocyanins). In order for the aroma precursors to develop the cocoa aroma, the cocoa beans must undergo roasting processes. Alkalization during roasting process diminishes the heterocyclic compounds of pyrones and furaneol in cocoa beans (Ziegleder et al., 1991).

Supercritical fluid extraction (SFE) method has been widely used for the extraction of volatile components from plants due to its rapid, effective and solvent-free sample pre-treatment technique (Pourmortazavi, et al. 2007). SFE is highly recommended as an extraction method because it does not leave chemical residue, provide solvent-free product and the CO_2 gas can be recycled and used again as part of the unit operation (Otles, et al. 2009). Determination of optimal condition in SFE is influenced, greatly, by operating temperature and pressure especially in critical region (Gomes, 2007), although other parameters such as sample particle size, flow rate of SFE solvent, extraction time and operation mode have some affects. The two former parameters change the density and viscosity of the SFE solvent mimicking the properties of various conventional solvent as in phase diagram of CO_2 (Figure 1). It was shown that by maintaining certain temperature of extraction and varying the pressure, or vice versa, several density of extracting solvent (CO_2) can be achieved resulting various compounds can be expelled out of the samples, including fat, aromatic compound and alkaloids. Most aromatic and flavor compounds have molecular weight below 250, which are soluble in SF CO_2 (Gomes, 2007) and normally can be extracted at low pressure where SF CO_2 has low density (Reverchon, 1997).

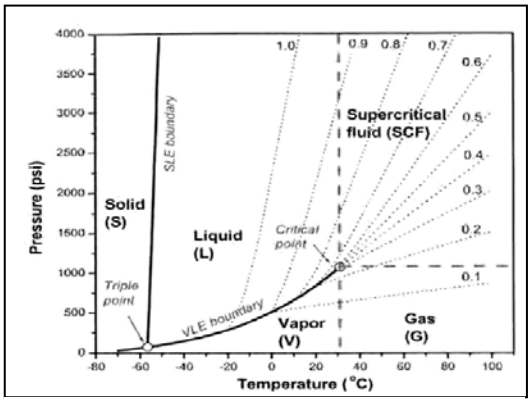

Figure 1: Density of SF CO2 by Temperature & Pressure (Anonymous, 2012)

This method had successfully extracted sesame seed oil (Corso, et al. 2010) and palm kernel oil (Zaidul, et al. 2007). Nevertheless, cocoa butter, polyphenols and pyrazines from cocoa were also successfully extracted *via* this method as reported by Saldana, et al. (2002); Sarmento, et al. (2008) and Sanagi, et al. (1997), respectively. Moreover, Sanagi et al (1997) conducted SFE at 200bar 60^{o}C with modifier to completely extract the pyrazines compounds. Meanwhile, Mohamed, et al. (2002) used high pressure of CO_2 and ethane at more than 152 bar resulting high recovery of cocoa butter, caffeine and theobromine compounds from cocoa beans. In addition, research on SFE cocoa butter was in depth as carried out by Asep, et al. (2008) and as mentioned. Ducki, et al (2008) conducted a research on using solid-phase micro-extraction (SPME) for headspace analysis in cocoa products for identification of cocoa aromatic compounds. SPME coupled with GC-FID was also used by Hasny (2012) in his research work on detection of aromatic compound from roasted cocoa beans. Despite all, most of cocoa aromatic compounds were reported based on study using conventional extraction. Research reports focusing on extraction of cocoa aromatic compound using SFE for perfumery application was also not in extensive. Fragrance application of cocoa extracted using SFE was also scarcely documented (King and Bott, 1993). Therefore this research was carried out to extract the aromatic compound from roasted cocoa beans using SFE in order to develop natural cocoa-scented perfume.

Materials and Procedure

Sample preparation

Fermented cocoa beans were collected from Cocoa Research and Development Centre at Jengka, Malaysia. A latest clone of MCB, namely MCB C1, was selected in this preliminary study, whereas other clones will be

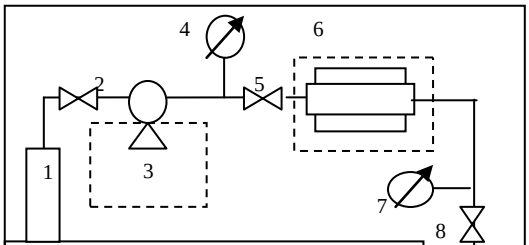

Figure 2: SFE Unit
1. Liquid CO_2 tank 2. Valve
3. Pump with chiller 4 .Flow rate indicator
5. Valve 6. Extraction vessel with insulator
7. Pressure indicator 8. Valve
9. Collection vessel

subjected to the next phase of extraction. The beans were then roasted in the oven at 135°C for 15 minutes. After shell removal, the beans were ground using warring blender and sieved to 1.0-0.5mm mesh size. Ground roasted beans were kept in tight container until extraction using SFE.

Supercritical Fluid Extraction

Extraction was carried out using SFE unit comprise of Intelligent HPLC Pump Model PU980 (Jasco Corporation, Tokyo, Japan) attached to cooling unit (Haake Fison DC 3). To achieve low temperature (-20°C), the cooling unit was operated with mixture of ethylene glycol-water (50:50 v/v). Ground cocoa beans at 10.15±0.16 grams were poured into sew white cloth and loaded into 25ml stainless steel tube (Thar Design, Inc., CL1043). To maintain temperature required, the column was inserted into Column Oven Model CO-960 (Jasco Corporation, Tokyo, Japan). Extraction pressure was controlled using Back Pressure Regulator (BPR) Model BP 880-81. Extraction was carried out using purified liquid carbon dioxide (CO_2) obtained from Linde Malaysia Sdn. Bhd. The used gas was dispersed into the air and the liquid sample was collected in a vials. Figure 2 is the illustration of SFE Unit.

Experimental Design

Multilevel factorial design was chosen for this study prior to optimization. At this stage, two factors were considered, namely pressure (bar) and time of extraction (min). There are four levels for each factor, resulting multilevel factorial design as in Table 2. Sixteen runs of experiments were carried out according to the standard order. Constant temperature at 35±2°C was selected in this study, where the density of SF CO_2 was manipulated (Figure 1). Responses measured were the amount of aromatic, fatty acid and alkaloid compound which represented by 5-methyl-2-phenyl-2-hexenal (cocoa hexenal), hexadecanoic acid and caffeine, respectively. In addition, yield of the extract was also considered.

Table 2: Parameters & Responses

	Parameters		*Responses*				*Total compounds detected*
Run Order	*Pressure (bar)*	*Time (min)*	*Yield (%)*	*Aromatic (mg/g)*	*Fatty acid (mg/g)*	*Alkaloids (mg/g)*	
1	125	45	0.70	5.25	85.05	7.70	19
2	200	75	1.46	8.01	307.28	13.38	16
3	100	75	0.57	5.41	78.63	14.53	17
4	125	75	0.79	9.97	339.38	531.21	8
5	200	45	1.20	24.50	396.22	467.93	15
6	150	75	1.29	18.76	288.46	254.83	18
7	100	60	0.19	2.56	15.96	24.33	18
8	100	45	0.19	2.25	22.65	36.27	14
9	125	60	0.39	6.19	77.12	68.33	19
10	200	60	1.34	14.81	344.62	208.65	18
11	125	30	0.30	4.35	21.75	21.15	19
12	200	30	0.89	10.6	62.19	111.94	16
13	150	60	1.19	14.87	197.42	122.50	13
14	150	45	0.80	11.18	163.27	94.61	16
15	100	30	0.20	0.88	7.32	3.51	14
16	150	30	0.69	6.18	55.3	51.86	21

Determination of compound in the extract

Absolute extract was transferred into gas chromatography (GC) vials by rinsing using 2.5ml n-hexane (Merck) followed by 2.5ml ethanol (HmbG). The calculation for amount of compound was based on the percentage area of each compound from the total yield of extract multiplying by the dilution factor (1:5) based on the solvent used to rinse the extract. Higher amount of compound extract indicated potential pressure and time to be used in further experimental works. Prior to GC-Mass Spectrophotometric (GC-MS) identification, the crude extract was filtered using C18-SepPak column (Supelco) with acetonitrile as the carrier to obtain clean extract. One µl sample was injected into the GC-MS system (Agilent Tech 7890A GC system). The GC-MS system has split-splitless column injector with mass spectrum detector (Agilent Tech System 5975C). Column of HP5MS (Agilent J&W) with dimension 30m x 0.25m i.d x 0.25µm was used in detection of compound. The condition was set at 220°C, 6.544 bar with helium as gas carrier. Library of National Institute of Standards & Technology (NIST 08) was used.

Statistical analysis

Minitab software Release 14 was used for statistical design and data analysis in this study. For this study, all the results were categorized as significant when the p-value is less than 0.05. The values were reported by mean.

Results and Discussion

From the statistical analysis, the pressure and time of extraction were significantly affecting the yield, at p=0.000 and p=0.001, respectively. However, no interaction between the parameters was obtained. The linear model equation obtained, using linear regression analysis, for yield is, YIELD =-1.16+0.00954*PRESSURE+0.0105*TIME (R^2=0.8700). It was indicated from this equation that yield was proportional to the increasing of pressure at nearly ten times in ratio with the time of extraction. The main effect plot for yield was plotted in Figure 3. In addition, pressure at 200 and 150bar has higher effect to the yield in comparison with the lower pressures, which however affecting at longer extraction time.

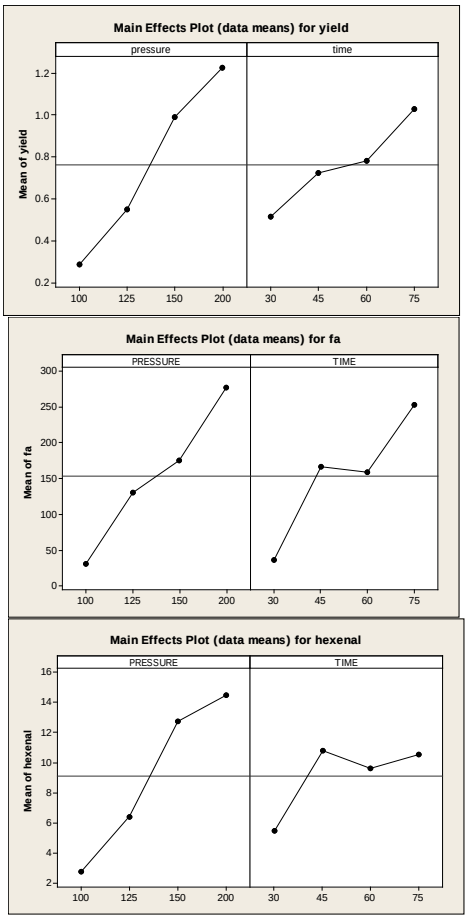

Figure 3: Plots for yield, fatty acid (fa) and aromatic (hexenal) compounds

Pressure and time of extraction was significantly affecting the amount of fatty acid compound extracted in this study at p-value of 0.015 and 0.032, respectively. The main effects were plotted separately in Figure 3 as there is no interaction between the two factors. Among these factors, only pressure has significant effect to the amount of aromatic compound extracted (p=0.022), whereas alkaloids compound was not significantly affected. By referring to Figure 1, the density of SF CO_2 was identified according to operation pressure and temperature. As the temperature was constant, the density was proportional with the pressure. Density of SF CO_2 in this study was 700, 725, 815 and 900kg/m^3, which significantly affected the yield and amount of aromatic compounds at p=0.001 and 0.015, respectively (at 35±2°C).

The amount of fatty acid compounds extracted by this method was significantly higher than the aromatic compounds. Reverchon (1997) reported that this is due to the SF CO_2 behavior of lipophilic solvent, but with adjustable selectivity, which extract lipid compound easily. It is also regarding to the position of paraffin and waxes substances on the surface and surrounding the vacuoles in plant materials which make its easily extracted using SFE. In addition, fat content in cocoa beans is more than 50% of the total weight of dry beans. Therefore, it was not surprising that extraction at any pressure point will result certain amount of fatty acid in the extract. There are two possible solutions worth trying at to overcome this condition, first, is by additionally using serial separators at different temperature and pressure are recommended to be applied to fractionate paraffin and waxes from aromatic compound extracted using SFE. Secondly, the extraction can be carried out at low pressure with high temperature to obtain low SF CO_2 density, where low molecular weight compounds, especially the aromatic compounds, can be extracted (Gomes, 2007).

Although the responses in this study was focusing on three compounds representing the

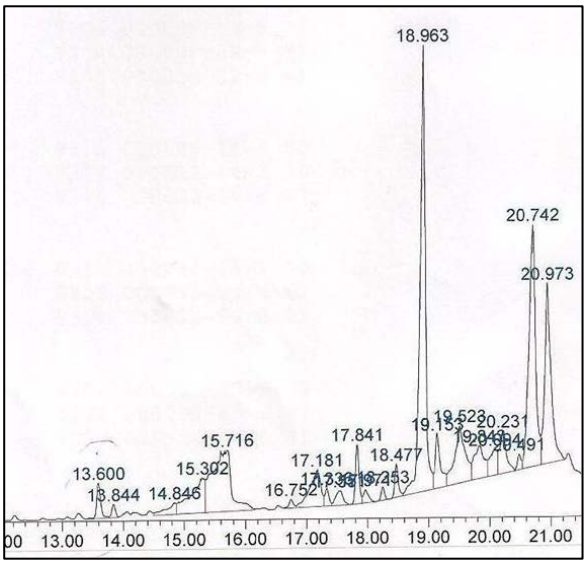

Figure 4: GC-Chromatogram for run 5

numbers of compounds that being identified using GC-MS, there are few aromatic compounds mentioned in Table 1 were not detected in the extract. For example, Ziegleder (1997) mentioned that high amount of linalool, contributed to the flowery and tea-like fragrance in flavor grade cocoa beans, which however absent in extract of cocoa bean in this study. Another key compound, 2,3,5-trimethyl-6-butylpyrazine was only detected at run 5 (pressure 200 bar, time of extraction 45 minutes). Low pyrazines compound was expected as alkalization process was omitted in sample preparation. Alkalization process is an essential step in cocoa processing to develop various color of cocoa powder and enhance the sensorial properties of final cocoa products. Figure 4 was the example of chromatogram for run 5. Other compounds listed in Table 1 were detected in each run (not discussed here). Total numbers of compounds detected for each run was listed in Table 2.

Conclusion

Cocoa aromatic compound can be extracted at 200bar with time of extraction for 45minutes, which however resulting higher amount of fatty acid compounds which is interference in this study. To use this condition, the SFE unit has to be modified with the addition of serial separators to fractionate the extract. On the other hand, lower pressure can be implemented coupled with higher temperature to create $SFCO_2$ with low density that can expel out the aromatic compound that has low molecular weight. The progress on this research is underway to perform these methods in order to obtain high yield of cocoa aromatic compound for formulation of natural cocoa-scented perfume.

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