ELSEVIER

Journal of Chromatography A, 785 (1997) 361–367

JOURNAL OF CHROMATOGRAPHY A

Supercritical fluid extraction of pyrazines in roasted cocoa beans Effect of pod storage period

M. Marsin Sanagi[a,*], Wong Pik Hung[a], Suhaimi Md Yasir[b]

[a]*Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 80990 Johor Bahru Johor, Malaysia*

[b]*Chemistry and Technology Division, Lembaga Koko Malaysia, 88999 Kota Kinabalu Sabah, Malaysia*

Abstract

Selected cocoa beans samples which had been subjected to various pod storage periods prior to fermentation were analysed for pyrazines. Carbon dioxide supercritical fluid extraction was used for the extraction of the compounds and quantitative and qualitative analyses of the extracts were achieved by using gas chromatography and gas chromatography–mass spectrometry. Pyrazine compounds identified in the extract included pyrazine, 2-methylpyrazine, 2,3-dimethylpyrazine, 2,6-dimethylpyrazine, trimethylpyrazine and tetramethylpyrazine. The concentration of pyrazine compounds in the cocoa beans, in particular 2,6-dimethylpyrazine and tetramethylpyrazine were found to be largely proportional to pod storage period. © 1997 Elsevier Science B.V.

Keywords: Cocoa beans; Pyrazines

1. Introduction

Cocoa (*Theobroma cacao* L.) is an economically important plant widely cultivated in countries of the equatorial belt including Africa, South America and Asia [1,2]. Malaysia is one of the leading producers of cocoa in the world. In 1990, there was an estimated 360 000 hectares of cocoa plantation in Malaysia and cocoa accounts for the third largest exported raw agricultural based commodity [3,4].

Procedures used by the cocoa processing industry follow many different paths depending on the characteristics of the final products. The cocoa pods can be stored for a few days after which they are split open as practised in Ghana [5]. Alternatively, fresh cocoa pods can be split open and the wet beans are subjected to modification (alteration) prior to fermentation such as pressing [6], yeast treatment, or bean spreading [7]. The pod storage is one of the preconditioning methods which effectively serves to reduce the pulp and water contents in the cocoa beans and increases micro-aeration within the pulp. This would increase the sugar respired by yeast rather than fermentation and eventually reduce alcohol fermentation and acetic acid formation [8]. The fresh beans are fermented by one of the several methods and subsequently dried. The next series of processes include roasting, crushing and winnowing (to removes germ and shells). The roasted cocoa nibs can further be fine-milled to form cocoa liquor which can be pressed to form cocoa butter and cocoa cakes.

Pyrazines are a group of important flavour compounds responsible for the cocoa taste [9]. Typical examples of pyrazines commonly found in cocoa products are pyrazine, 2,5-dimethylpyrazine and tetramethylpyrazine. The quality of chocolate products is largely dependent on the origin and genotype of cocoa, curing processes (pod storage, fermen-

*Corresponding author.

0021-9673/97/$17.00
PII S0021-9673(97)00569-4

tation, drying, etc.), roasting conditions and grinding processes of the cocoa beans [1]. The quantity of pyrazines in cocoa beans has been shown to be directly dependent on these processes [1,10,11]. Quality control is therefore important in the production of cocoa products such as chocolate, with tastes that are desirable to the consumers and thus, a supply of quality cocoa is very important in the cocoa-related industries.

Supercritical fluid extraction (SFE) has proved to be suitable for the extraction of various substances including lipid-bearing materials [12]. SFE of cocoa has been included in a British Patent in 1974 [13]. By following a similar experimental set-up, Vitzthum et al. [14] extracted cocoa beans from Ghana by SFE and obtained a 20% yield of yellowish cocoa butter with intense cocoa odour. More recently, Rossi et al. [15] described the SFE of cocoa liquor and nibs using CO_2 as the extractant to yield 99% and 74% of extracts, respectively. However, little work has been reported on the extraction of pyrazines from cocoa using SFE.

In this study, the pyrazines contents of selected Malaysian cocoa beans samples which had been subjected to various pod storage periods prior to fermentation were analysed. Carbon dioxide SFE was carried out for the extraction of the compounds. Quantitative and qualitative analyses of the extracts were achieved by using gas chromatography and gas chromatography–mass spectrometry (GC–MS).

2. Experimental

2.1. Materials

Methanol (HPLC grade) was from J.T. Baker, Phillipsburg, NJ, USA. Standard pyrazine compounds: pyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine and tetramethylpyrazine (Aldrich Chemical, Milwaukee, WI, USA) and fermented dried cocoa beans were gifts from the Malaysian Cocoa Board, Kota Kinabalu, Sabah, Malaysia. Liquid CO_2 was industrial grade from Malaysian Oxygen (MOX, Petaling Jaya, Selangor, Malaysia).

2.2. Instrumentation

The SFE apparatus consisted of a Jasco PU-890 Intelligent HPLC pump (Jasco Corporation, Tokyo, Japan) fitted with a cooling jacket to deliver CO_2. In order to cool the pump head, ethylene glycol–water mixture (50:50, v/v) was circulated through the cooling jacket using a Grant CC-20 chiller and circulator which could deliver coolant down to −15°C. A 3-ml or 10-ml extraction cell (Keystone Scientific, Bellafonte, PA, USA) was used as the extraction vessel. A Rheodyne 7125 or a Rheodyne 7037 valve (Cotati, California, USA) was used to effect switching between the load and extract positions. A Shimadzu CTO-6A oven (Kyoto, Japan) or a Gow Mac GC oven (Madison, NJ, USA) was used to maintain a constant extraction temperature of 60°C. A Jasco 880-81 back-pressure regulator was used to control the extraction pressure. A 50-ml tapered test tube with an L-shaped side arm was used as the collection vessel.

GC analyses on the cocoa extracts and standard pyrazine compounds were performed using a HP5880 gas chromatograph on a HP-20M (Carbowax 20M) column (25 m×0.32 mm×0.3 μm film thickness) (Hewlett–Packard, Avondale, USA) with He as the carrier gas at 1.85 ml/min, oven temperature program 50–200°C at 6°C/min, a final time of 10 min.

GC–MS analyses were carried out using a HP5890 Series II gas chromatograph in conjunction with a HP5989A mass spectrometer with electron impact ionisation at 70 eV and ionisation source temperature of 176°C. The GC conditions were maintained as in the GC analysis. Peak identification was based on mass spectra search and comparison (Wiley Library 3.1).

2.3. Procedures

Solutions of each of the standard pyrazines (~2000 ppm) were prepared by dissolving the compounds in dichloromethane. Aliquots (100-μl) of the standard solutions were spiked onto glasswool matrix (laboratory grade) placed in the extraction vessel. Qualitative analysis of the compounds were based on gas chromatographic retention time comparison. Quan-

titative analyses were carried out by using *n*-octadecane ($C_{18}H_{38}$) as the internal standard.

2.4. Preparation of cocoa sample

Ripe pods were stored on the farms for periods of 0–2 days, 4–5 days and 9–10. After these periods, the pods were split open and the wet beans (~800 kg) were immediately loaded into shallow boxes for 120 h of fermentation with one turn after 48 h. After completing fermentation, the wet beans were artificially dried on a flat bed dryer with hot air temperature between 35–45°C until the moisture content was reduced to less than 7%.

Samples of dried cocoa beans (20 g) were roasted at 145°C for 40 min. After removing the shells, the nibs were ground in an electric blender to give nib powder. The samples were extracted and analysed within 3 days to minimise loss of volatile compounds.

The cocoa nib powder samples were extracted using a 10-ml extraction vessel which can accommodate up to 4.5 g of ground nibs without leaving much dead space. The samples were extracted at a pressure of 200 bar at 60°C in the static SFE mode for the first minute (with no outlet flow from the extraction vessel) followed by dynamic SFE for the next 30 min (with continuous flow of extractant). Static mode SFE was important since the modifier (e.g. methanol 2% v/v) was added directly onto the sample or matrix containing the standard compounds. This allowed thorough diffusion and mixing of the supercritical fluid CO_2 and the modifier with the sample matrix prior to the dynamic SFE [16,17].

2.5. Analyte trapping technique

In this work, a simple analyte trapping technique was used by depressurising the supercritical fluid into a collection vessel which was filled with solvent (e.g. dichloromethane) as the solvent trap. A length of stainless steel tubing (10 cm×1 mm I.D.) was attached to the outlet end of the back-pressure regulator. A 50-ml tapered test tube with an L-shape side arm was used as the collection vessel. The L-shape side arm (to vent off the CO_2 gas) was stuffed with some silanised glass wool for trapping the more volatile analytes. Dichloromethane (2 ml) was used as the trapping solvent. To trap the volatile components which might escape from the solvent trap, the collection vessel was further cooled by placing it in an ice bath or a glass-coil heat exchanger through which coolant (ethylene glycol–water 50:50, v/v) was passed. In this way, the collection vessel could be cooled to about −8°C.

3. Results and discussion

3.1. Optimisation of method

Optimisation of the SFE method was carried out using three pyrazine standards, namely pyrazine, 2,5-dimethylpyrazine and tetramethylpyrazine. The percentage analyte recoveries were determined by varying the parameters of interest.

3.1.1. Effect of trapping solvent

As the analyte solubility is dependent on the SFE trapping solvent, four solvents of different polarity and boiling points were examined for their efficiency as trapping solvents in of SFE of pyrazine compounds (Table 1). This method was chosen as it was relatively simple, inexpensive to perform and the extracts were immediately ready for chromatographic analysis using conventional injection techniques. It was found that solvents showed varying efficiencies. In general, dichloromethane and acetone were found to give better analyte recovery than methanol or *n*-hexane. The former solvents showed almost complete recoveries for 2,5-dimethylpyrazine and tetramethylpyrazine. Although acetone showed a slightly higher recovery for pyrazine than dichloromethane, the latter was selected as the trapping solvent in the later part of this work as it has a lower boiling point (39.8°C) than acetone (56.2°C) and is thus more favourable for the analyte pre-concentration step prior to chromatographic analysis.

The effect of the depth of the trapping solvent which allowed a length of the outlet stainless steel SFE tubing be immersed was investigated using two collection vessels of different sizes. It was found that with the same amount of solvent (10 ml dichloromethane), a depth of 5.5 cm using a collection vessel of

Table 1
Effect of trapping solvent on percent recovery of analytes

Trapping solvent	Percent recovery (%)		
	Pyrazine	2,5-Dimethylpyrazine	Tetramethylpyrazine
Dichloromethane	71.4	100.3	98.5
Acetone	82.4	103.2	98.5
n-Hexane	0	91.7	92.1
Methanol	42.1	86.0	66.7

SFE conditions: CO_2 extractant at 60°C, 200 bar, trapping solvent 10 ml, height 5.5 cm, dynamic SFE 30 min following 1-min static SFE.

1.6 cm I.D. showed better trapping efficiencies for the test compounds (pyrazine 71.4%; 2,5-dimethylpyrazine 100.3%; tetramethylpyrazine 98.5%) than those with a depth of 2.0 cm using a wider collection vessel of 3.2 cm I.D. (pyrazine, 65.9%; 2,5-dimethylpyrazine, 71.7%; tetramethylpyrazine, 92.1%).

3.1.2. Effect of pressure

An experiment was set up to examine the effect of pressure on the SFE recovery of pyrazines (Table 2). The SFE temperature was maintained at 60°C. It was found that at a sub-critical pressure of 60 bar, SFE of the analytes gave very low recoveries of the pyrazines, in particular, tetramethylpyrazine which has the highest molecular weight of the three compounds. The percent recoveries of the analyte increased at a SFE pressure of 100 bar and started to level off at pressures of 150 bar and 200 bar.

3.1.3. Effect of extraction time

The analyte recoveries with the variation of the collection period were also investigated. The results showed that large percentages of pyrazine and 2,5-dimethylpyrazine were collected in the first 15 min of extraction. Meanwhile, a large percentage of tetramethylpyrazine was collected in the time period of 16–30 min after the commencing of SFE. The variation of the total recovery of the analytes with extraction time is shown in Fig. 1.

3.1.4. Effect of modifier

Because of its low polarity, carbon dioxide is unable to readily extract polar analytes. It has been previously reported that an addition of a polar modifier to the CO_2 can increase the extraction

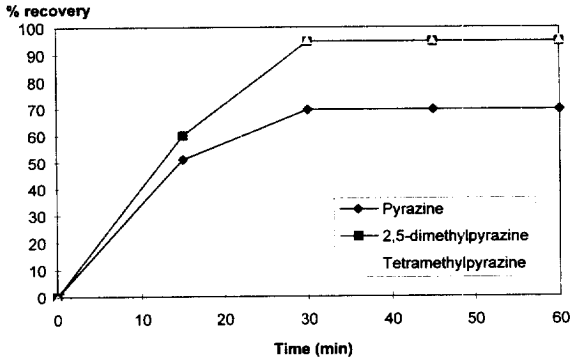

Fig. 1. Effect of extraction time on total recovery of analytes. SFE conditions: CO_2 extractant at 60°C, 150 bar, trapping solvent: dichloromethane (10 ml, 5.5 cm depth).

Table 2
Effect of pressure on the recovery of pyrazines[a]

Pressure (bar)	Recovery (%)		
	Pyrazine	2,5-Dimethylpyrazine	Tetramethylpyrazine
60	23.8	28.7	0
100	27.5	71.7	66.7
150	78.7	100.3	102.8
200	71.4	100.3	98.5

[a] SFE conditions: CO_2 extractant at 60°C, 200 bar, trapping solvent: dichloromethane (10 ml, depth 5.5 cm), dynamic SFE 30 min following a 1-min static SFE.

recovery of the more polar analytes as a result of an increased dissolving power and ability of the supercritical fluid to compete for the active sites on the matrix [11]. As incomplete recoveries of the analytes, particularly pyrazine, were experienced when using pure carbon dioxide as the extractant, experiments were performed to investigate the effect of modifier.

The modifier was introduced by pipetting a specific amounts of the modifier and dropping the modifier directly onto the sample in an extraction vessel before the vessel was quickly closed and reattached to the SFE system. It has been reported previously [16,17] that this method of introduction of modifier in SFE is economical, easy to perform and poses less mechanical or reproducibility problems compared to methods where the modifier is introduced by means of a second pump and a mixer [18] or using premixed extractant from a cylinder [16].

Methanol and dichloromethane were used at different proportions (Table 3). It was found that the pyrazine recoveries increased with the increase in proportions of dichloromethane (0% to 2% and 5%) while complete recoveries of 2,5-dimethylpyrazine and tetramethylpyrazine were generally maintained. Marked increase in the recovery of pyrazine was observed (86%) on adding 2% methanol as the modifier. However, the recovery decreased when 5% methanol was used. This was probably due to the negative effect of increased percentage of modifier on the trapping efficiency of analytes [16]. These results showed that 5% dichloromethane and 2% methanol as the modifier were able to give reasonably good recoveries of the standard compounds on glasswool matrix with the former showing slightly better recovery for pyrazine. However, preliminary SFE experiments on a sample of roasted cocoa beans using 2% methanol and 5% dichloromethane as the modifiers showed that 2% methanol was able to more readily extract the pyrazines and thus this method was used in subsequent analysis of roasted cocoa beans.

Table 4
The concentration of pyrazine compounds in cocoa samples with different pod storage periods

Pyrazine compounds	Pyrazine compounds concentrations in roasted cocoa beans (ppm)		
	Pod storage period		
	0–2 days	4–5 days	9–10 days
Pyrazine	–	6	11
2,5-Dimethylpyrazine	–	–	Trace
2,6-Dimethylpyrazine	148	164	186
Tetramethylpyrazine	550	683	932

3.2. Analysis of pyrazines from roasted cocoa beans

Three cocoa samples which had undergone different pod storage period of up to 2 days, 4–5 days and 9–10 days, respectively, were analysed for their pyrazines content (Table 4). The selected SFE conditions were CO_2 modified with methanol (2% v/v) at 200 bar, 60°C, 1 min static SFE followed by 30 min dynamic SFE, extract collected in dichloromethane (10 ml, 5.5 cm depth). Pyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, trimethylpyrazine and tetramethylpyrazine were identified in the extract by GC–MS (Fig. 2). The relative concentrations of the three pyrazine compounds, namely pyrazine, 2,6-dimethylpyrazine and tetra-

Table 3
Effect of modifier on analyte recovery

Modifier[a]	Recovery (%)		
	Pyrazine	2,5-Dimethylpyrazine	Tetramethylpyrazin
Pure carbon dioxide	78.8	100.3	102.8
Methanol (2% v/v)	86.0	100.3	101.7
Methanol (5% v/v)	78.7	91.7	95.3
Dichloromethane (2% v/v)	73.2	100.3	95.3
Dichloromethane (5% v/v)	93.3	103.2	102.8

[a] Volume per volume. SFE conditions: CO_2 extractant at 60°C, 150 bar, trapping solvent: dichloromethane (10 ml, 5.5 cm depth), dynamic SFE 30 min following 1 min static SFE.

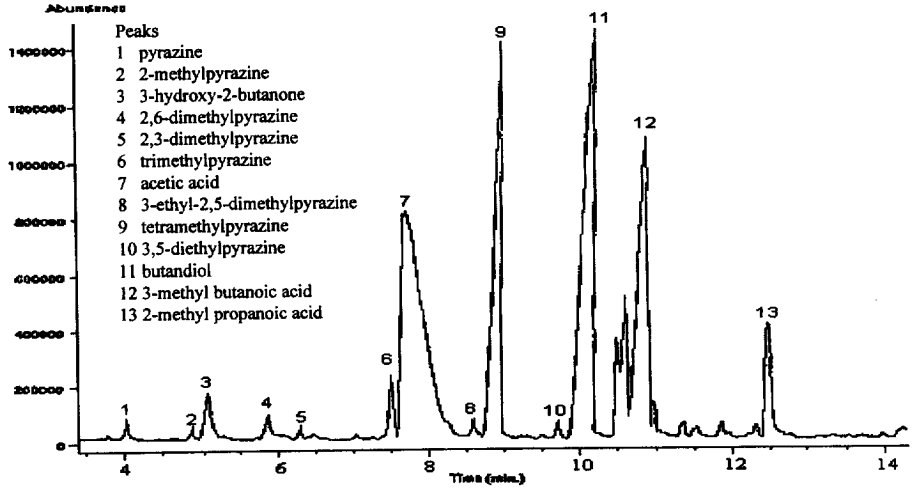

Fig. 2. Partial GC–MS total ion chromatogram of the separation of a cocoa sample (T18) SFE extract. SFE conditions: CO_2 modified with methanol (2% v/v) at 200 bar, 60°C, 1 min static SFE followed by 30 min dynamic SFE, extract collected in dichloromethane (10 ml, 5.5 cm depth). GC conditions: column HP-20M (Carbowax 20M) (25 m×0.32 mm×0.3 μm film thickness) carrier gas He at 1.85 ml/min, oven temperature program 50–200°C at 6°C/min, final time 10 min.

methylpyrazine increase with pod storage period (Table 4). In general, the results are within the expected ranges. The pyrazine and tetramethylpyrazine contents are higher than those reported for unroasted cocoa beans by Humbert and Sandra [9] who used micro-version Likens–Nikerson steam distillation–extraction method (pyrazine 0 ppm; 2,6-dimethylpyrazine 1.7 ppm; tetramethylpyrazine 4.85 ppm). Reineccius et al. [19] employed a solvent extraction and fractionation method followed by GC to analyse Ghana roasted cocoa beans and reported higher tetramethylpyrazine concentration values which are equivalent to 1630–2414 ppm. Hashim and Chaveron [20] used coupled steam distillation–microdistillator method to analyse roasted cocoa beans from Ivory Coast and reported tetramethylpyrazine concentrations which ranged approximately from 1700–4300 ppm.

Amongst the three samples analysed, the sample with 10-day pod storage gave the highest levels of all the three pyrazines (Table 4). Amongst the three pyrazines, the amount of pyrazine was found to be lowest. This could be attributed to the greater loss in the collection, concentration and analytical processes. The quantity of pyrazine compounds recovered by SFE generally increased with pod storage period from 2 through 5 and to 10 days. Clapperton et al. [10] reported an increase in the cocoa aroma and a reduction in astringent and sour taste with an increase in pod storage period. This result, however, contradicts a report by Baker et al. [11] who suggested the use of short pod storage periods for the production of chocolate products which are more acceptable to a taste panel.

4. Conclusions

Carbon dioxide supercritical fluid extraction has been successfully used for the extraction of the pyrazine compounds in roasted cocoa beans. By using a GC method, eight pyrazine compounds were identified in the extract. These include pyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, tetramethylpyrazine and trimethylpyrazine. The concentration of pyrazine compounds in the cocoa beans, in particular 2,6-dimethylpyrazine and tetramethylpyrazine were found to be largely proportional to pod storage period.

Acknowledgements

We thank Mr. Ayob and Mr. Abd Kadir for

technical assistance in the GC–MS analyses. This work was supported in parts by Universiti Teknologi Malaysia and the National Science Development and Research Council (MPKSN) through the IRPA Programme No. 2-03-08-002-2.1.

References

[1] G.A.R. Wood, R.A. Lass, Cocoa, Wiley, New York, 1989.
[2] Kursus dalam Khidmat: Tanaman Koko/Kelapa, Jabatan Pertanian Negeri Perak dan MARDI, Government Printing Department, Perak, Malaysia, 1977, pp. 29–33.
[3] Economic Report 1983/84-1992/93, Ministry of Finance Malaysia, National Printing Department, Kuala Lumpur, Malaysia.
[4] Laporan Tahunan MARDI 1990 (Annual Report), Malaysia Institute of Agricultural Research and Development (MARDI), Kuala Lumpur, Malaysia, 1991, pp. 31–33.
[5] B. Meyer, B. Biehl, M.B. Said, J.R. Samarakoddy, J. Sci. Food Agric. 48 (1989) 285.
[6] C.F. Chong, R. Shepherd, Y.C. Poon, Mitigation of Cocoa Bean Acidity: Fermentry Investigation, Proc. Int. Conf. on Cocoa and Coconuts, Kuala Lumpur, 1978, pp. 387–414.
[7] B. Biehl, B. Meyer, M.B. Said, J.R. Samarakoddy, J. Sci. Food Agric. 51 (1990) 35.
[8] M. B, Said, B. Meyer, B. Biehl, Pulp preconditioning: A New Approach Towards Quality Improvement of Malaysian Cocoa Beans. Proc. MARDI Senior Staff Conf., Kuala Lumpur, Malaysia, 1988, pp. 28–57.
[9] B. Humbert, P. Sandra, LC–GC 5 (1985) 1035.
[10] J. Clapperton, S. Yow, J. Chan, D. Lim, R. Lockwood, L. Romanczyk, J. Hammerstone, Trop. Agric. 71 (1994) 303.
[11] D.M. Baker, K.I. Tomlins, C. Gay, Food Chem. 51 (1994) 425.
[12] S.A. Westwood, SFE and Its Use in Chromatographic Sample Preparation, Blackie and Professional, Glasgow, 1993.
[13] HAG AG. 1974, Method of producing Cocoa Butter, Brit. Pat. 1.356.750.
[14] O.G. Vitzthum, P. Werkhoff, P. Hubert, J. Food Sci. 40 (1975) 911.
[15] M. Rossi, C. Arnoldi, F. Antoniazzi, A. Schiraldi, Italian Food Beverage Technol. II (1993) 28–33.
[16] S. Bowadt, S.B. Hawthorne, J. Chromatogr. 703 (1995) 549.
[17] V. Camel, M. Caude, A. Tambute, J. Chromatogr. Sci. 33 (1995) 123.
[18] M.M. Sanagi, U.K. Ahmad, R.M. Smith, J. Chromatogr. Sci. 31 (1993) 20.
[19] G.A. Reineccius, P.G. Keeney, W. Weissberger, J. Agric. Food Chem. 20 (1992) 202.
[20] L. Hashim, H. Chaveron, Food Res. Int. 27 (1994) 537.