Chapter 7

Pyrazines Formation by *B. subtilis* TN51 During *Thua Nao* Fermentation

7.1 Introduction

Thua Nao, widely consumed in the northern part of Thailand is a conventional fermented soybean generally used as a flavour enhancer in various dishes. It is mainly fermented by *Bacillus subtilis* and related bacilli (Chukeatirote *et al*., 2006). Usually, *Thua Nao* has a unique odour due to the presence of several volatile components during fermentation, and increased concentration of volatiles by means of enzymatic activities from microorganism suggested by Sugawara *et al*. (1985), Owens *et al*. (1997), Tanaka *et al.* (1998) and Leejeerajumnean *et al*. (2001). Moreover, Strecker degradation and Maillard reactions are the most important for generation of volatile compounds such as pyrazines, aldehydes, alcohols, acids, esters, and other compounds in the final product (Shu, 1998; 1999; Larroche *et al*., 1999).

The unique olfactory sensation is the primary character of the fermented soybeans relating to quality of the products. For instance, *Natto*-like odour or fruity/nutty aroma indicates a good quality product, while ammonia-like and fishy odour specify unpleasant odour of food (Sugawara *et al*., 1985; Tanaka *et al*., 1998; Leejeerajumnean *et al*., 2001). Numerous reports have suggested various profiles of volatile compounds derived from fermented and non-fermented soybeans, such as 1-octen-3-ol, hexanal, hexanol, pentanol, 2,5-dimethylpyrazine, 2-ethyl-6-methylpyrazine, 2-pentylfuran, 2-ethylfuran, and benzaldehyde indicated liberating roasted, nutty, greeny and beany odour of cooked non-fermented soybeans (Sugawara *et al*., 1985; Owens *et al*., 1997; Dakwa *et al*., 2005). Whilst the major volatile compounds presented in fermented soybean derived from different groups. For instance, Tanaka *et al*. (1998) found ethanol, acetone, 2,3-butanedione, 2-methyl-3-pentanone and 2,3-pentanedione in Korean *Chungkukjang*, as well as Owens *et al*. (1997) found a large amount of 3-hydroxy-2-butanone, 2,5-dimethylpyrazine and trimethylpyrazine during fermentation of soy-*Daddawa*. In addition, Leejeerajumnean

et al. (2001) found 25 volatiles in traditional *Thua Nao* with the highest contents being of 3-hydroxy-2-butanone, 2-methylbutanoic acid, pyrazines, dimethyl disulfide, and 2-pentylfuran. Sugawara *et al*. (1985) and Tanaka *et al*. (1998) detected different major components in pure-starter culture *Natto* such as 2,5-dimethylpyrazine, 2-methylbutanoic acid, acetone, 2,3,5-trimethylpyrazine and 2,3-butanedione. Ouoba *et al*. (2005) also noted that highest contents of pyrazines in African *Soumbala,* fermented by pure starter *B. subtilis* obtained significant preferred score than those naturally fermented products, having greater aldehyde contents. The correlation between volatiles and sensory attributes in Korean *Doenjang* has also been evaluated by Lee and Ahn (2009), who found that furfuryl alcohol and maltol were highly associated with sweet-grain attribute.

A previous study reported the volatiles of traditional *Thua Nao* fermented by mixed natural organisms (Leejeerajumnean *et al*., 2001), but the volatiles in pure-starter fermentation have not been yet studied. Therefore, to improve the organoleptic quality and safety of traditional *Thua Nao* which usually contaminated with spoilage and pathogenic organisms, this investigation intended to identify these compounds produced by pure starter *B*. *subtilis* in comparison with naturally fermented product as well as the non-fermented soybean.

7.2 Materials and methods

7.2.1 Extraction of volatile compounds

Bacillus subtilis TN51 was prepared as starter culture for soybean fermentation as described in Section 4.2.2 to 4.2.3. Fermented soybeans were collected after 72 h-fermentation, smashed and stored at -20°C until analysed. All frozen *Thua Nao* and their cooked non-fermented soybeans (CNF) were thawed overnight at 4°C prior to analysis. Soybean paste samples (5 g) were then placed in 15 ml screw-capped amber glass vials (Supelco, Bellefonte, PA, USA). Twenty microliter of 1,2-dichlorobenzene (100 mg/l in water), as an internal standard, was added to the sample before extraction. Solid phase microextraction (SPME) fiber, carboxen/polydimethylsiloxane (carboxen/PDMS) (75 μm, Supelco, Inc., Bellefonte, PA, USA) was used for the extraction of volatiles components. Vials containing

samples were pre-equilibrated for 30 min at 60°C in a thermostatic water bath. Subsequently, the SPME fiber was inserted into the headspace above the soybean sample for adsorption the headspace volatiles for 30 min (Qin and Ding, 2007). After extraction, the loaded SPME fiber was removed immediately from the sample vial and inserted into the injection port of a gas chromatography (GC) for further analysis. Triplicate extractions were prepared for each sample.

7.2.2 Analysis of volatile compounds

The extracted volatiles were desorbed from the SPME fiber by heating at 300°C for 5 min in the injection port of a Hewlett-Packard (HP) gas chromatography model 5890 with a HP-Innowax capillary column (60 m x 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies, Inc., USA) while cooling the column to 0°C by dried ice. The detector was a mass spectrometer (HP 5972 Mass Selective Detector, Hewlett-Packard, Bracknell, UK). The chromatography run was started by warming the column to 40°C at 4°C/min, held at 40°C for 5 min, heated to 260°C at a rate of 4°C/min and held at 260°C for further 20 min. The flow rate of helium carrier gas was 1.8 ml/min. The mass spectrophotometer was operated in the electron impact mode with ion source temperature, 200°C, ionisation voltage, 70 eV, ionisation current, 100 μA and accelerating voltage, 2 kV. The mass range was 29 - 400 amu with a scanning rate of 1.44 scans/sec. Identity confirmation of volatile compounds was proceeded by non-polar column, VF-5ms (60 m x 0.25 mm i.d., 0.25 μm film thickness) using the same condition as described above, except the final temperature adjusted to 300°C.

7.2.3 Identification and quantification of volatile compounds

The GC-MS was typically calibrated by running 0.1 μl of 100 ppm standard mixture of C_5-C_{25} *n*-alkanes. Quality of volatile compounds were identified by comparing linear retention indices (LRI) with that of authentic compounds, mass spectra of compounds compared with those of the bibliographic data of known compounds from the mass spectral database. Approximate quantity of each volatile compound was manipulated by integration of peaks of the total ion chromatogram and

calculated by measuring its peak area compared with that of 1,2-dichlorobenzene internal standard.

7.2.4 Statistical analysis

Data were expressed as means ± standard error of mean of triplicate observations. The data were also subjected to analysis of variance (ANOVA) and *t*-test, and compared differences by Duncan's multiple range test at $P \leq 0.05$ level of significance.

7.3 Results and discussion

The typical GC chromatograms and contents of volatile compounds in cooked non-fermented soybeans (CNF) and *Thua Nao* are shown in Figure 7.1 and Table 7.1. The autoclaved soybean (CNF2) displayed 3 times higher amount of total volatiles than boiled soybean (CNF1). Furthermore, the profile of autoclaved soybean comprised 42 volatile components including 9 alcohols, 4 aldehydes, 6 ketones, 3 aromatics, 4 acids and esters, 3 pyrazines, 4 furans, 2 sulphur-containing, and 7 miscellaneous compounds. On the other hand, boiled soybean showed only 36 volatile components including 9 alcohols, 4 aldehydes, 4 ketones, 4 aromatics, 3 acids and esters, a pyrazine, 4 furans, 2 sulphur-containing, and 5 miscellaneous compounds. Boiled soybean exhibited the largest proportion of ketones (36.09%) (Figure 7.2A) with the major volatile carbonyl compounds comprised of ethanol, 2,3-butanedione, 3-hydroxy-2-butanone and acetone. For autoclaved soybean, the highest proportion of volatile compounds was alcohols (54.01%) (Figure 7.2B), with the most abundant compounds being ethanol, acetone, benzaldehyde, and 2,5-dimethylpyrazine. Other investigators also found ethanol as one of the major volatiles in cooked non-fermented soybeans (Ruth *et al*., 2005; Dakwa *et al*., 2005). More aromatic and Maillard components occurred in this product, since most of these compounds are found in heated foods. The volatiles such as pyrazines, furans, aldehydes, aromatic compounds, pyrrole and thiazole which produce from Strecker degradation in Maillard reactions were identified in cooked soybeans released nutty, roasted or bready aroma of heated foods (Reineccius, 2006).

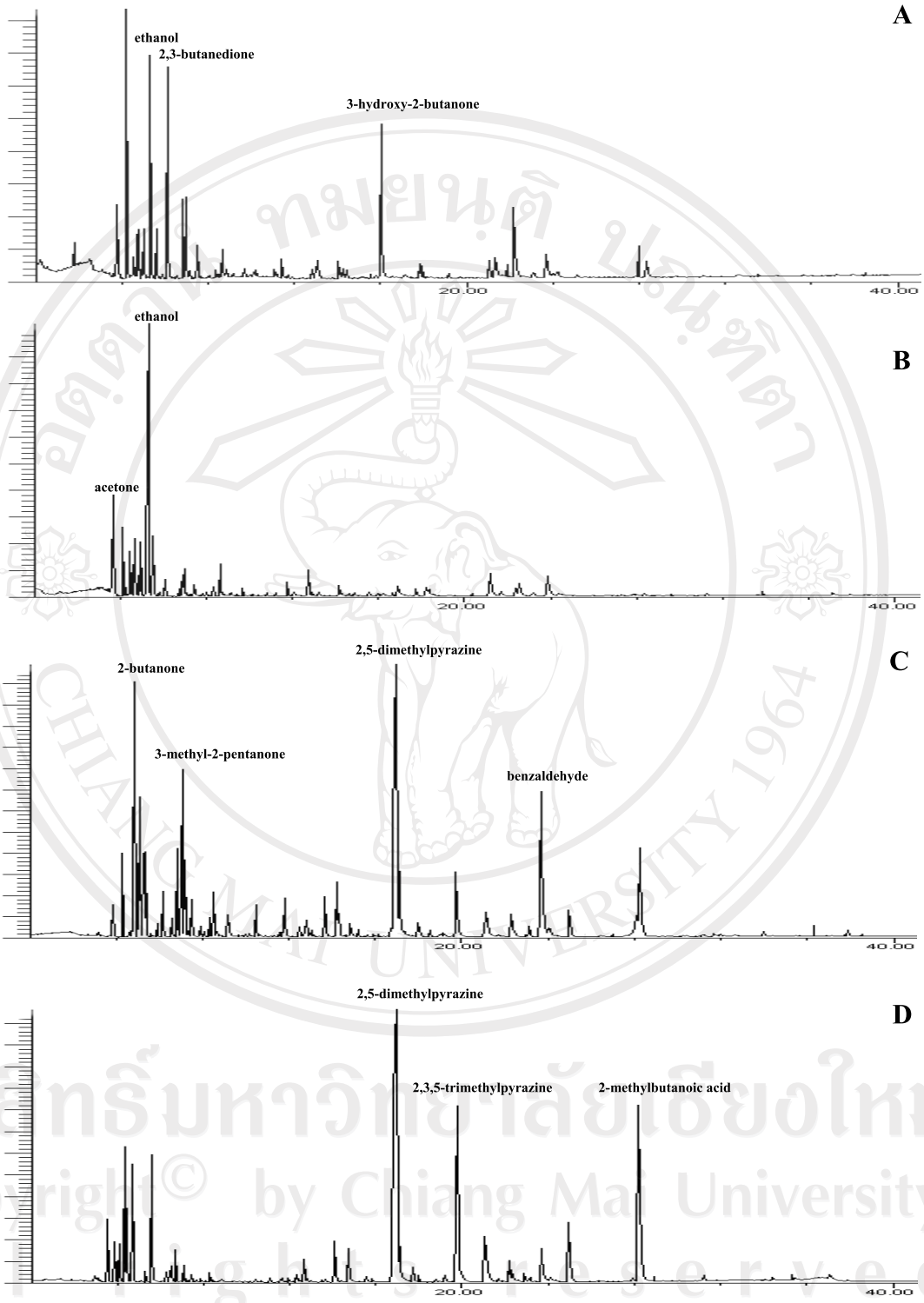

Figure 7.1 SPME-GC-MS Chromatograms of flavour volatile compounds: A, boiled non-fermented soybeans (CNF1); B, autoclaved non-fermented soybeans (CNF2); C, *Thua Nao* produced by natural fermentation (TNMX); D, *Thua Nao* produced by pure starter culture of *Bacillus subtilis* TN51 (TNB51).

Table 7.1 Concentration of flavour volatile compounds (mg/kg dry basis) in cooked non-fermented soybeans (CNF) and *Thua Nao* products

Volatile compound[1]	Exp. LRI.[2]		Cooked non-fermented soybean		Fermented soybean (*Thua Nao*)[3]	
	HP-Innowax	VF-5ms	CNF1	CNF2	TNB51	TNMX
Alcohols						
Isopropyl alcohol	924		-	-	-	5.37±2.12
Ethanol	894	<600	9.77±2.51^{b}	68.28±8.89^{a}	16.90±3.54^{b}	4.14±1.94^{b}
2-Butanol	997		-	-	2.00±1.45^{b}	6.12±1.15^{a}
Propanol	1001		0.23±0.05^{b}	0.98±0.10^{ab}	0.46±0.05^{ab}	2.42±1.94^{a}
2-Methylpropanol	802	<600	-	-	0.55±0.26^{b}	3.60±1.86^{a}
1-Penten-3-ol	1138		0.11±0.02^{b}	0.39±0.04^{ab}	0.70±0.17^{a}	0.21±0.04^{b}
1-Butanol	1132	645	0.93±0.23^{a}	0.44±0.06^{b}	-	-
2-Methyl-1-butanol	1184	732	0.49±0.26^{a}	2.03±0.20^{a}	1.92±0.62^{a}	1.16±73^{a}
3-Methyl-1-butanol	1186	729	0.91±0.30^{c}	3.84±0.34^{a}	2.91±0.77^{ab}	1.89±0.29b^{c}
3-Methyl-2-pentanol	1181		-	-	-	0.95±0.22
1-Pentanol	1001		0.54±0.11^{b}	1.38±0.27^{a}	-	-
3-Methyl-3-buten-1-ol	1226	725	-	-	6.00±1.75^{a}	5.87±1.41^{a}
Hexanol	1331	867	0.68±0.16^{b}	1.09±0.08^{b}	2.78±0.61^{a}	0.11±0.02^{b}
(Z)-3-Hexen-1-ol	1359		-	-	0.48±0.12	-
1-Octen-3-ol	1428	980	0.86±0.26^{b}	3.19±0.08^{a}	3.33±0.57^{a}	1.68±0.53^{b}
Total alcohols (15)			**14.53±3.49^{b}**	**81.62±9.61^{a}**	**37.70±8.57^{b}**	**33.50±11.74^{b}**
Aldehydes						
2-Methylpropanal	802	<600	-	-	0.89±0.06	-
2-Methylbutanal	866	642	0.84±0.15^{c}	3.06±0.16^{b}	8.89±1.07^{a}	3.74±0.70^{b}
3-Methylbutanal	872	640	2.39±0.39^{c}	4.68±0.19^{c}	14.83±1.09^{a}	8.51±1.15^{b}
Hexanal	1053	797	1.32±0.03^{b}	3.06±0.56^{a}	0.46±0.14^{b}	1.24±0.28^{b}
2-Methyl-2-butenal	1061	736	-	-	0.32±0.06	-
Benzaldehyde	1508	969	2.58±0.18^{c}	6.86±0.79^{c}	15.50±3.16^{b}	42.73±3.26^{a}
Total aldehydes (6)			**7.13±0.75^{d}**	**17.67±1.66^{c}**	**40.73±3.94^{b}**	**56.21±2.52^{a}**
Aromatic compounds						
Benzene	930	644	0.17±0.01^{a}	-	-	0.12±0.03^{a}
Ethylbenzene	1094	860	0.42±0.10^{a}	0.60±0.36^{a}	-	-
Styrene	1226	893	1.23±0.29^{a}	1.08±0.17^{a}	-	-
1-Methyl-2-isopropylbenzene	1235	1027	0.39±0.06^{a}	0.27±0.03^{b}	0.15±0.01^{c}	0.11±0.02^{c}
Total aromatic compounds (4)			**2.08±0.27^{a}**	**1.96±0.57^{a}**	**0.15±0.01^{b}**	**0.23±0.04^{b}**
Ketones						
Acetone	754	<600	4.11±0.83^{ab}	10.04±2.71^{a}	1.29±0.05^{b}	5.63±1.01^{ab}
2-Butanone	851	<600	2.49±0.60^{c}	4.90±0.39^{bc}	9.43±2.20^{b}	33.12±2.39^{a}
3-Methyl-2-butanone	885	645	-	-	0.99±0.03^{b}	4.04±1.07^{a}
2,3-Butanedione	933		7.35±0.77^{a}	2.77±0.26^{a}	12.23±6.34^{a}	-
2-Pentanone	972	667	-	-	-	4.55±0.48
3-Methyl-2-pentanone	977	730	-	-	3.34±0.03^{b}	19.98±0.43^{a}
2-Methyl-3-pentanone	995	724	-	-	-	17.34±7.96
4-Methyl-2-pentanone	967	727	-	0.18±0.03^{b}	1.01±0.05^{b}	7.15±1.72^{a}
2,3-Pentanedione	1020	687	-	-	0.33±0.09^{a}	0.41±0.18^{a}
5-Methyl-3-hexanone	1054	828	-	-	-	36.41±3.80
5-Methyl-2-hexanone	1112	852	-	-	0.85±0.08^{b}	8.65±1.14^{a}
6-Methyl-3-heptanone	1196	967	-	-	-	6.93±0.67

Table 7.1 (*continued*)

Volatile compound[1]	Exp. LRI. [2]		Cooked non-fermented soybeans		Fermented soybeans (*Thua Nao*)[3]	
	HP-Innowax	VF-5ms	CNF1	CNF2	TNB51	TNMX
6-Methyl-2-heptanone	1207	953	-	-	0.58±0.07^{b}	5.83±0.41^{a}
5-Methyl-2-heptanone	1227	963	-	-	1.03±0.02^{b}	3.78±0.36^{a}
3-Octanone	1233	985	-	-	0.85±0.32^{b}	4.48±0.81^{a}
3-Hydroxy-2-butanone (acetoin)	1271	726	6.13±2.29^{a}	0.64±0.07^{b}	0.66±0.41^{b}	0.05±0.01^{b}
1-Octen-3-one	1275	977	-	0.42±0.06^{ab}	0.67±0.10^{a}	0.30±0.08^{b}
Total ketones (17)			**20.09±3.58^{b}**	**18.90±2.14^{b}**	**32.08±4.26^{b}**	**144.04±18.47^{a}**
Acids and Esters						
Methyl acetate	718	<600	-	-	0.55±0.24	-
Ethyl acetate	814	601	0.79±0.06^{a}	3.57±0.35^{a}	3.35±1.63^{a}	-
Methyl 2-methylpropanoate	872	673	-	0.17±0.03^{b}	1.34±0.08^{a}	1.05±0.20^{a}
Ethyl 2-methylpropanoate	913	750	-	-	1.43±0.16	-
Methyl 2-methylbutanoate	958	770	-	0.25±0.10^{b}	2.24±0.18^{a}	-
Methyl 3-methylbutanoate	966	763	-	-	0.79±0.05^{b}	1.32±0.06^{a}
Ethyl 2-methylbutanoate	1009	851	-	-	1.17±0.08^{a}	0.77±0.13^{b}
Ethyl 3-methylbutanoate	1018	850	-	-	0.43±0.08	-
Acetic acid	1431	654	1.90±0.18^{b}	3.26±0.72^{b}	17.54±5.28^{a}	3.07±1.02^{b}
Propanoic acid	1520	722	-	-	0.57±0.12^{a}	0.30±0.08^{a}
2-Methylpropanoic acid	1547	807	-	-	18.28±3.89^{a}	3.96±0.78^{b}
2-Methylbutanoic acid	1649	902	1.09±0.59^{b}	-	63.40±13.23^{a}	15.02±3.98^{b}
Total acids and esters (12)			**3.42±0.66^{b}**	**7.11±0.98^{b}**	**111.09±23.76^{a}**	**25.23±6.24^{b}**
Pyrazines						
Pyrazine	1192	729	0.19±0.02^{b}	0.71±0.24^{ab}	1.00±0.24^{a}	0.36±0.04^{b}
2-Methylpyrazine	1245	825	-	0.86±0.29^{b}	7.12±1.64^{a}	4.25±0.86^{ab}
2,5-Dimethylpyrazine	1308	918	-	6.84±3.95^{b}	147.82±36.86^{a}	103.61±10.69^{a}
2,6-Dimethylpyrazine	1312		-	-	13.00±3.21^{a}	9.80±1.39^{b}
2,3-Dimethylpyrazine	1331	926	-	-	1.09±0.43^{a}	0.11±0.01^{b}
2-Ethyl-5-methylpyrazine	1372		-	-	2.02±0.41^{a}	0.65±0.10^{b}
2-Ethyl-6-methylpyrazine	1366	1002	-	-	0.31±0.03^{a}	0.18±0.03^{b}
2,3,5-Trimethylpyrazine	1388	1006	-	-	43.76±13.57^{a}	10.84±1.34^{b}
2-Ethyl-3,5-dimethylpyrazine	1432	1080	-	-	0.94±0.22^{b}	1.44±0.40^{a}
Tetramethylpyrazine	1460	1088	-	-	3.72±1.60	-
Total pyrazines (10)			**0.19±0.02^{b}**	**8.41±4.48^{b}**	**220.79±57.94^{a}**	**131.26±12.71^{a}**
Furans						
Tetrahydrofuran	745	615	-	-	1.52±0.36	-
2-Methylfuran	836	<600	0.24±0.04^{b}	0.62±0.10^{a}	0.16±0.03^{b}	0.50±0.15^{ab}
2-Ethylfuran	910	690	2.06±0.38^{b}	5.07±0.23^{a}	0.17±0.03^{c}	0.10±0.03^{c}
2-Vinylfuran	1041		0.44±0.14^{b}	0.73±0.16^{a}	-	-
2-Pentylfuran	1195	989	0.50±0.02^{b}	2.28±0.02^{a}	0.34±0.07^{c}	0.31±0.03^{c}
Total furans (5)			**2.92±0.38^{b}**	**7.95±0.70^{a}**	**2.18±0.24^{bc}**	**0.90±0.19^{c}**

Table 7.1 (*continued*)

Volatile compound[1]	Exp. LRI.[2]		Cooked non-fermented soybeans		Fermented soybeans (*Thua Nao*)[3]	
	HP-Innowax	VF-5ms	CNF1	CNF2	TNB51	TNMX
Sulphur-containing compounds						
Dimethyl disulfide	1034	734	0.14±0.02^{b}	0.39±0.05^{b}	0.86±0.47^{b}	2.22±0.53^{a}
Dimethyl trisulfide	1351	975	0.02±0.00^{b}	0.10±0.01^{b}	0.23±0.11^{b}	0.47±0.10^{a}
Total sulphur-containing compounds (2)			**0.15±0.02^{b}**	**0.49±0.05^{b}**	**1.09±0.58^{b}**	**2.70±0.43^{a}**
Miscellaneous						
1,3-Octadiene	911	821	-	0.37±0.01^{a}	0.08±0.02^{b}	0.11±0.02^{b}
Chloroform	988	608	2.75±0.26^{a}	1.58±0.30^{b}	-	-
Thiophene	991	651	0.59±0.23^{b}	2.88±0.34^{a}	-	-
Toluene	1004	762	1.70±0.28^{a}	1.28±0.11^{a}	0.52±0.09^{b}	0.39±0.06^{b}
Pyridine	1154	742	-	-	1.91±0.59	-
Trimethyl-Oxazole	1165	845	-	-	1.46±0.30	-
Thiazole	1211		-	0.82±0.06^{b}	-	1.19±0.14^{a}
2,6-Dimethylpyridine	1229		-	-	0.19±0.02	-
Pyrrole	1491		0.18±0.05^{c}	0.30±0.05^{c}	1.04±0.18^{b}	1.66±0.19^{a}
Acetamide	1748	823	-	-	1.20±0.47^{a}	0.39±0.06^{b}
o-Methoxyphenol	1843	1092	-	-	0.22±0.06^{b}	0.43±0.20^{a}
Maltol	1952	1118	-	-	1.21±0.53	-
Phenol	1983	986	0.12±0.02^{b}	0.18±0.03^{b}	0.29±0.07^{b}	1.24±0.20^{a}
Indole	2419		-	-	-	2.98±0.29
Total miscellaneous (14)			**5.15±0.69^{a}**	**7.01±1.02^{a}**	**7.95±1.34^{a}**	**7.40±0.75^{a}**
Total volatile compounds (85)			**54.24±9.27^{b}**	**147.66±16.52^{b}**	**452.68±93.47^{a}**	**389.40±7.51^{a}**

Data are mean ± standard error of mean (n = 3). Means in the same row with different small letters are significantly different ($P \leq 0.05$); (-), not detectable.

[1] Volatiles were identified based on mass spectrum and linear retention index consistent with those of an authentic standard.

[2] Experimental linear retention index on HP-Innowax and VF-5ms columns.

[3] CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, *Thua Nao* produced by fermentation of boiled soybeans with naturally occurring microbes in; TNB51, *Thua Nao* was prepared by fermentation of autoclaved soybeans with *B. subtilis* TN51

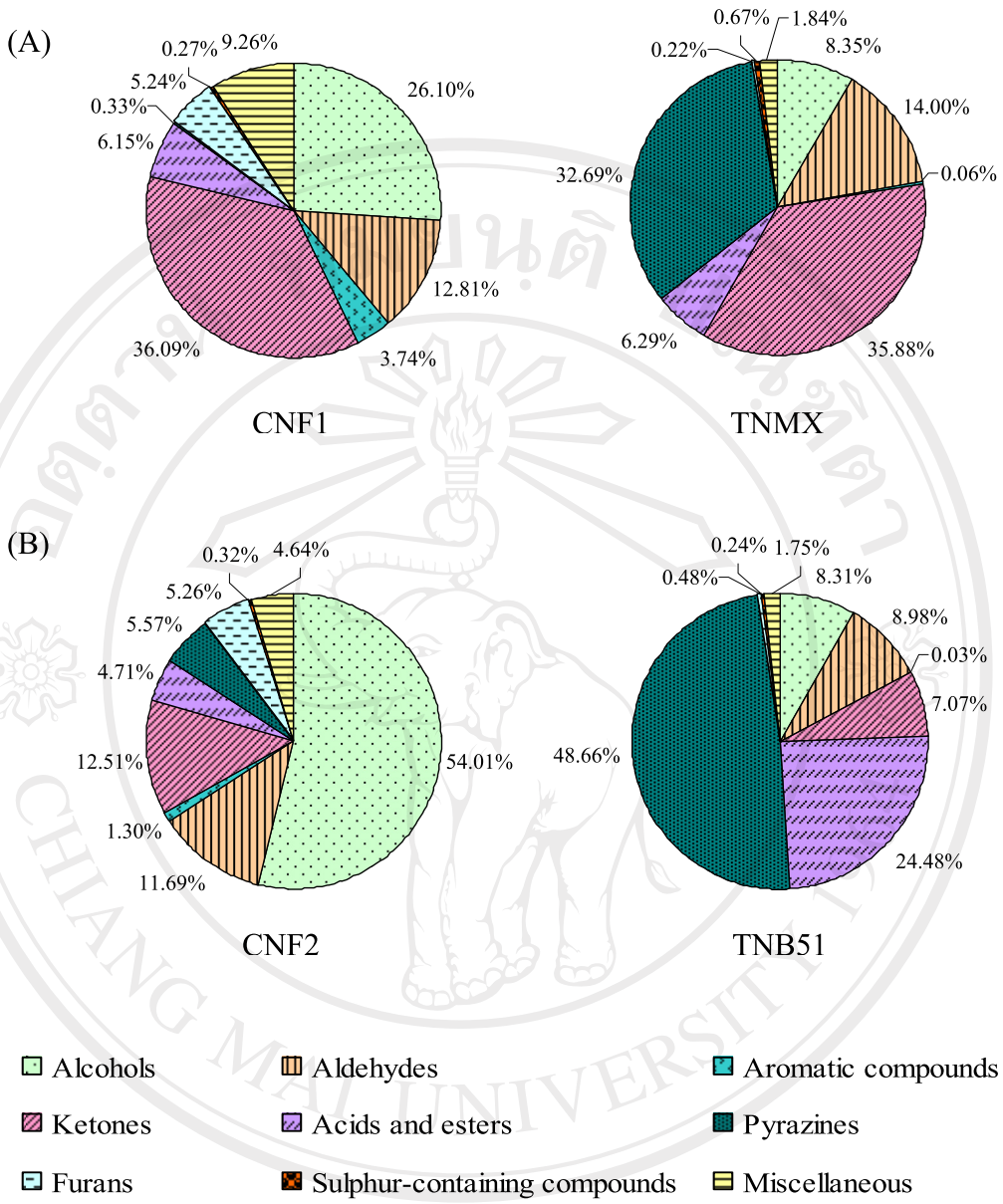

Figure 7.2 Proportion of flavour volatile compounds in naturally (A) and *B. subtilis* TN51 fermented *Thua Nao* (B). CNF1, CNF2 = cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, *Thua Nao* was prepared by fermentation of boiled soybeans with naturally occurring microbes; TNB51, *Thua Nao* was prepared by fermentation of autoclaved soybeans with *B. subtilis* TN51.

The volatile carbonyl compounds have strong impact on odour of the product because of their sensitivity to olfactory receptors (Grosch, 1982). Whilst Sugawara *et al*. (1985) reported that hexanal, hexanol, 2-pentylfuran, and 1-octen-3-ol contributed to greenness, musty and beany odour of soybeans. Most of these compounds were also identified in both boiled and autoclaved soybeans which exceeded their threshold values, suggesting that they might liberate from autoxidation and enzymatic oxidation of unsaturated fatty acids plus Strecker degradation and Maillard reactions (Kato *et al*., 1981; Reineccius, 2006). Enzymatic oxidation of these products might come from the interaction of lipoxygenase and its substrate linoleic and linolenic acid (Guss *et al*., 1967).

Variation derivative of pyrazines could be identified from cooked soybeans. For instance, Owens *et al*. (1997) found 10 pyrazine derivatives in autoclaved soybean and only 3,6-dimethyl-2-ethylpyrazine presented at higher amount than their threshold odour. Similar results were obtained in this study, 3 pyrazine derivatives, pyrazine, 2-methylpyrazine and 2,5-dimethylpyrazine, were identified in autoclaved soybean and only 2,5-dimethylpyrazine presented at concentration over threshold value which presumably contributed grassy, nuts, and roasted odour (Guadagni *et al*., 1972). In addition, pyrazine and methylpyrazine were the products of serine degradation, while 2,5-dimethylpyrazine derived from threonine (Shu, 1999).

Furans are usually derived from heated carbohydrates through Maillard reactions. Several investigators had identified 2-pentylfuran in boiled and autoclaved soybeans (Sugawara *et al*., 1985; Owens *et al*., 1997; Leejeerajumnean *et al*., 2001). Sugawara *et al*. (1985) also suggested that 2-pentylfuran was a key compound of beany odour. In this study, styrene and benzaldehyde were found in both cooked soybeans which were complied with result obtained from Owens *et al*. (1997), whereas Leejeerajumnean *et al*. (2001) found only benzaldehyde in 7 h boiled soybeans. However, all volatiles found in cooked non-fermented soybeans were at much lower concentration that those present in fermented soybeans (Table 7.1).

Table 7.1 illustrates that many volatiles were identified after fermentation. Generally, pyrazines, sulphur-containing compounds, acids and esters, ketones, aldehydes and miscellaneous compounds were present in high amounts, whereas aromatics and furans were found at very low concentration in these fermented

products. Pyrazines (691 times) and sulphur-containing compounds (18 times) were the most increased in naturally fermented TNMX, while the most abundant volatiles in TNB51 fermentation were pyrazines (26 times) and acids and esters (16 times). Similar results have also been reported in traditional *Thua Nao* (Leejeerajumnean *et al*., 2001) and *Daddawa* (Owens *et al*., 1997) for an increase of ketones, pyrazines, acids and esters, and sulphur-containing compounds. Many volatiles were identified only in fermented *Thua Nao* including 6 alcohols, 2 aldehydes, 11 ketones, 7 acids and esters, 7 pyrazines, a furan, and 7 miscellaneous. Hence, it can be concluded that these compounds were produced by microbial transformation. Total volatile compounds increased from its original non-fermented form 7 and 3 times in *Thua Nao* produced by natural (TNMX) and *B. subtilis* TN51 fermentation (TNB51), respectively. However, the concentration of total volatiles in both *Thua Nao* were not significantly different ($P > 0.05$), but this does not mean that these two categories comprised the same amount of individual volatiles. Further characterisation of each individual volatile could be quantified as follows. Volatiles detected from TNB51 *Thua Nao* comprised of 11 alcohols, 6 aldehydes, 13 ketones, an aromatic, 12 acids and esters, 10 pyrazines, 4 furans, 2 sulphur-containing, and 10 miscellaneous compounds, whereas TNMX *Thua Nao* consisted of 12 alcohols, 4 aldehydes, 16 ketones, 2 aromatics, 7 acids and esters, 9 pyrazines, 3 furans, 2 sulphur-containing, and 8 miscellaneous compounds.

The major volatile compounds detected from pure *Bacillus*-fermented *Thua Nao* including 2,5-dimethylpyrazine, 2-methylbutanoic acid, 2,3,5-trimethylpyrazine, 2-methylpropanoic acid and acetic acid which accounted for 64% of the total compounds. In contrast, the most abundant volatiles found in the naturally fermented *Thua Nao* included 2,5-dimethylpyrazine, benzaldehyde, 5-methyl-3-hexanone, 2-butanone and 3-methyl-2-pentanone, which accounted for 60% of the total compounds. Pure starter fermented *Thua Nao* displayed the highest concentration of pyrazines (48.66%) and acids and esters (24.48%) (Figure 7.2B), while naturally fermented product showed the largest amount of ketones (35.88%) and pyrazines (32.69%) (Figure 7.2A). Pyrazines is indicated as the typical compounds of *Natto*-like odour or fruity, nutty aroma in *Natto* and *Chungkukjang* (Sugawara *et al*., 1985; Tanaka *et al.,* 1998). However, 6 of 10 pyrazines (i.e. 2,5-dimethylpyrazine, 2,6-

dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2-ethyl-5-methylpyrazine and 2-ethyl-6-methylpyrazine) found in present study that displayed such high concentration than those their threshold values (Karahadian and Johnson, 1993; Buttery *et al*., 1997). The higher content of pyrazines in pure *Bacillus*-fermented *Thua Nao* was 68% of that in naturally fermented product (Figure 7.3). Hence, those in *Thua Nao*, especially TNB51 are expected to contribute to the fruity and nutty aroma in the product. Recently, Lee and Ahn (2009) also detected 9 pyrazines in commercial *Doenjang* with the major components of 2,5-dimethylpyrazine, tetramethylpyrazine and trimethylpyrazine which related to its soy sauce attributes.

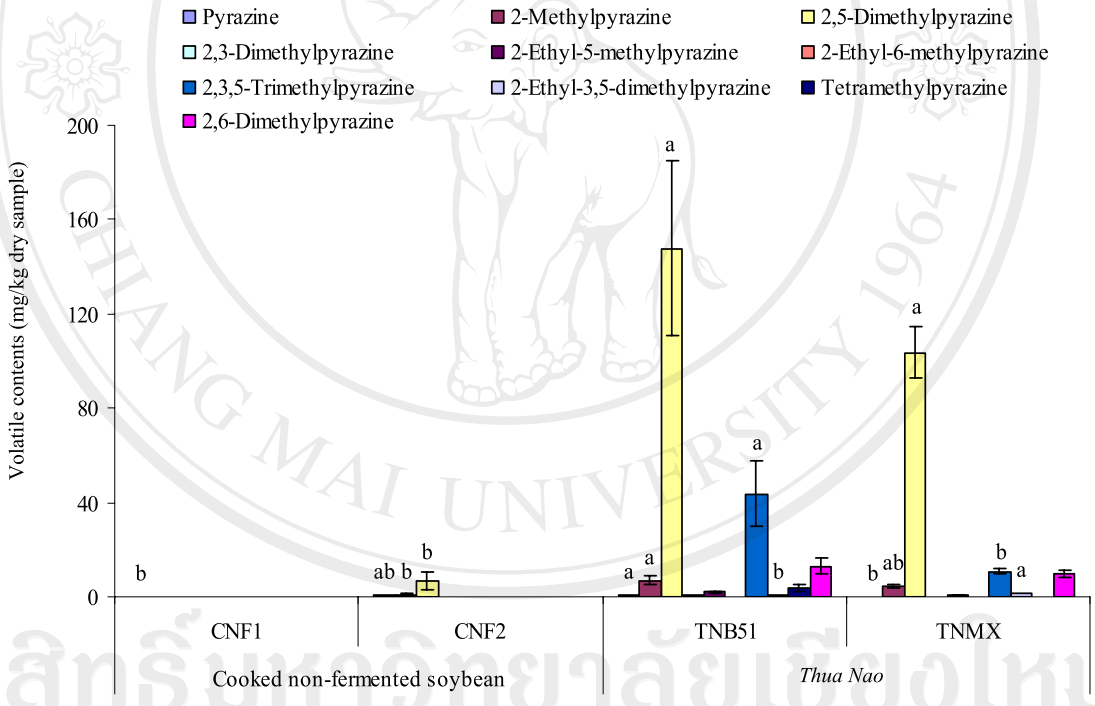

Figure 7.3 Pyrazines in cooked non-fermented soybeans and *Thua Nao*. CNF1, CNF2 = cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, *Thua Nao* was prepared by fermentation of boiled soybeans with naturally occurring microbes; TNB51, *Thua Nao* was prepared by fermentation of autoclaved soybeans with *B. subtilis* TN51. Each value represents mean ± SD (n = 3). Means (bar value) with different letters are significantly different ($P \leq 0.05$).

This study is inconsistent with the report of Leejeerajumnean *et al.* (2001), who reported that the most abundant volatiles of 3-hydroxy-2-butanone, 2-methylbutanoic acid, pyrazines, dimethyl disulfide, and 2-pentylfuran were present in naturally fermented *Thua Nao*. In addition, smaller profile (25 compounds) and content (3.5 mg/kg wet weight) of volatiles were also found in their report when compared with present study that detected 63 volatiles of 123.7 mg/kg wet weight in TNMX *Thua Nao*. These discrepancies may be because the differences method of extraction and type of GC column (Tenax GC trapping system and non-polar BPX-5 column for Leejeerajumnean *et al.* (2001) and SPME fiber extraction and polar HP-Innowax column for present study) were used.

Comparisons of the major volatiles between *Thua Nao* and other *Bacillus*-fermented foods are summarised in Table 7.2. Generally, predominant volatiles of these products are different. For instance, Owens *et al*. (1997) obtained a large amount of 3-hydroxy-2-butanone, 2,5-dimethylpyrazine, and trimethylpyrazine in 18 h fermented soy-*Daddawa* but these volatiles disappeared beyond 36 h incubation. In this study all compounds still retained even over 72 h fermentation time. The major volatiles detected in naturally fermented *Chungkukjang* were ethanol, acetone, 2,3-butanedione, 2-methyl-3-pentanone and 2,3-pentanedione (Tanaka *et al*., 1998) as well as acetoin, acetic acid and acetic acid ethyl ester in *Douchiba* (Qin and Ding, 2007). All these substances were also found in this study but in the lesser extent. The discrepancy between results is probably due to the different type of fermenting organisms, soybean cultivar, cooking process, fermentation time, extraction of volatiles and analysis method (Sugawara *et al*., 1985; Owens *et al*., 1997; Leejeerajumnean *et al*., 2001; Jeleń, 2003; Garcia-Esteban *et al*., 2004; Ouoba *et al*., 2005; Ikeda *et al*., 2006).

Table 7.2 Predominant flavour volatiles in *Bacillus*-fermented soybeans

Volatiles	*Thua Nao*			*Natto*[1, 3, 4]	*Chungkukjang*[3]	*Daddawa*[5]	*Douchiba*[6]
	TN[1]	TNMX[2]	TNB51[2]				
Alcohols	2-Butanol 2-Methyl-1-propanol	2-Butanol 3-Methyl-3-buten-1-ol Isopropyl alcohol	Ethanol 3-Methyl-3-buten-1-ol 1-Octen-3-ol	Ethanol 1-Octen-3-ol	**Ethanol** 1-Propanol 2-Methyl-1-propanol	1-Hexanol	2,3-Butanediol 1-Octen-3-ol
Aldehydes	**Benzaldehyde** 3-Methylbutanal 2-Methylbutanal	**Benzaldehyde** 3-Methylbutanal	Benzaldehyde 2-Methylbutanal	Nonanal Decanal	Benzaldehyde	**Benzaldehyde** Nonanal Decanal	Ethyanal 2-Phenylpropanal
Ketones	**Butanone** **3-Methylpentanone** **3-Hydroxy-2-butanone**	**5-Methyl-3-hexanone** **2-Butanone** **3-Methyl-2-pentanone** 2-Methyl-3-pentanone	2,3-Butanedione 2-Butanone	**Acetone** **3-Hydroxy-2-butanone**	**Acetone** **2,3-Butanedione** **2-Methyl-3-pentanone** **2,3-Pentanedione** 3-Hydroxy-2-butanone	**3-Methyl-2-pentanone** 2-Heptanone 3-Octanone	**3-Hydroxy-2-butanone** 2-butanone
Acids and esters	**2-Methylbutanoic acid**	2-Methylbutanoic acid	**2-Methylbutanoic acid** **2-Methylpropanoic acid** **Acetic acid**	Methyl isobutylate Acetic acid	2-Methylbutanoic acid Acetic acid 2-Methylpropanoic acid Ethyl propanoate Ethyl isobutyrate	Methyl 2- or 3-methylbutanoate Decyl butanoate	**Acetic acid** **Ethyl acetate**
Furans	**2-Pentylfuran**	2-Methylfuran 2-Pentylfuran	Tetrahydrofuran 2-Pentylfuran	3-Methylfuran	2,5-Dimethylfuran	2-Pentylfuran	2-Pentylfuran
Pyrazines	**2,5-Dimethylpyrazine** Trimethylpyrazine Tetramethylpyrazine	**2,5-Dimethylpyrazine** 2,3,5-Trimethylpyrazine 2,6-Dimethylpyrazine	**2,5-Dimethylpyrazine** **2,3,5-Trimethylpyrazine** 2,6-Dimethylpyrazine Tetramethylpyrazine 2-Methylpyrazine	**2,5-Dimethylpyrazine** **2,3,5-Trimethylpyrazine** **Tetramethylpyrazine** 2-Methylpyrazine	2,5-Dimethylpyrazine 2,6-Dimethylpyrazine	**Trimethylpyrazine** **Tetramethylpyrazine**	2,3,5-Trimethylpyrazine Tetramethylpyrazine 2,5-Dimethylpyrazine
Sulphur-containing compounds	**Dimethyl disulfide**	Dimethyl disulfide	Dimethyl disulfide	4-Ethyl-2-methylthiazole (E)-3,5-Dimethyl-1,2,4-trithiolane (Z)-3,5-Dimethyl-1,2,4-trithiolane Thialdine	-	Dimethyl disulfide	Propyl disulfide
Aromatic compounds	Benzyl alcohol	Benzene 1-methyl-2-isopropylbenzen	1-Methyl-2-isopropylbenzen	Ethylbemzine	-	**2-Methoxyphenol**	m-Xylene 2-Methoxyphenol
Miscellaneous	Indole	Indole	Pyridine	Thiophen	-	Trimethyloxazole Indolin	Indole

Source: [1]Leejeerajumnean *et al*. (2001); [2]present study; [3]Tanaka *et al*. (1998); [4]Sugawara *et al*. (1985); [5]Owens *et al*. (1997); [6]Qin and Ding (2007). TNMX, *Thua Nao* was prepared by fermentation of boiled soybeans with naturally occurring microbes; TNB51, *Thua Nao* was prepared by fermentation of autoclaved soybeans with *B. subtilis* TN51; (-), data not available; Main volatiles are bold.

The other the key compounds of nutty odour appeared to be associated with soybean fermented food was 2-methybutanoic acid (Tanaka *et al*., 1998). In our study, this acid derivative was also identified with significantly high concentration, i.e. 63.4 mg/kg (dry basis) in TNB51 *Thua Nao* and 15.02 mg/kg (dry basis) in TNMX sample (Figure 7.4). Since only little amount was found in boiled soybean (1.09 mg/kg dry basis), hence this could be confirmed that fermentation process gave rise to the formation of 2-methybutanoic acid. Leejeerajumnean *et al*. (2001) and Lee and Ahn (2009) also reported comparable data. Not only pyrazines were produced from free amino acids in *B. subtilis* fermented soybeans, but other organic acids including 2-methylbutanoic acid and 2-methylpropanoic acid were also detected after 3 days of fermentation as a result of catabolism of Leu and Ile (Larroche *et al*., 1999). Other volatile products such as ethanol, 2-butanol, 1-propanol, 1-butanol, 2-methylpropanol, 3-methylbutanol, 2-methylbutanol, benzaldehyde, 2-heptanone, 2-butanone, 3-methylbutanal, 2,3-butanedione, 3-hydroxy-2-butanone, 2-methylbutanoic acid, 2-methylpropionic acid, dimethyl disulfide, dimethyl trisulfide, ethyl acetate, styrene, chloroform, phenol, and indole could also be generated from amino acid metabolism (Tavaria *et al*., 2002; Muñoz *et al*., 2003). Acids and esters constituted the second major part of volatiles in TNB51 and presented significantly higher concentration than those found in the TNMX *Thua Nao*. Similar observations for high amount of alcohols and esters were also found in commercial *Sufu* (Chung, 1999) and *Doenjang* (Lee and Ahn, 2009). In this study, the apparent acids and esters such as, ethyl 2-methylpropanoate, ethyl 3-methylbutanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 2-hydroxy-4-methylpentanoic acid, propanoic acid and 2-methylpropanoic acid could only be detected from fermented soybeans. Several ethyl esters detected in *Thua Nao* were similar as found in commercial *Chungkukjang* and *Natto* (Tanaka *et al*., 1998). However, only ethyl 2-methylpropanoate was quantified above odour threshold values (Steinhaus and Schieberle, 2007) that may contribute little to fruity odour of TNB51.

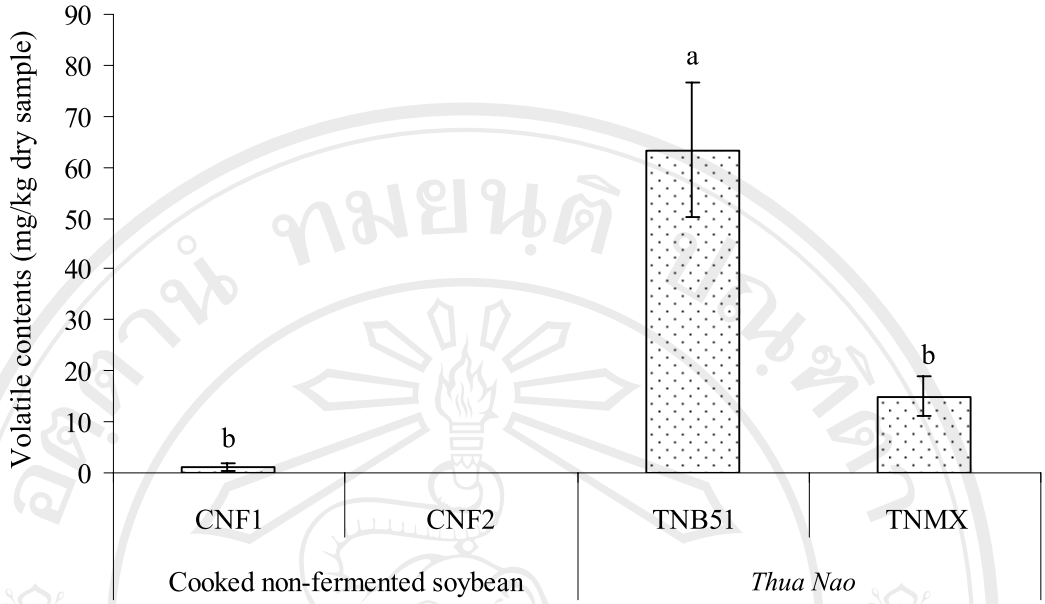

Figure 7.4 2-Methylbutanoic acid in cooked non-fermented soybeans and *Thua Nao*. CNF1, CNF2 = cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, *Thua Nao* was prepared by fermentation of boiled soybeans with naturally occurring microbes; TNB51, *Thua Nao* was prepared by fermentation of autoclaved soybeans with *B. subtilis* TN51. Each value represents mean ± SD (n = 3). Means (bar value) with different letters are significantly different ($P \leq 0.05$).

Table 7.1, 17 ketones were identified, among all derivatives 3 compounds presented at higher concentration than their odour threshold values, including 2,3-butanedione (buttery odour), 2,3-pentanedione (almond odour), and 1-octen-3-one (mushroom-like odour) (Stephan and Steinhart, 1999) (Figure 7.5). Ketones usually derived from lipids and amino acids degradation during microbial fermentation (Owens *et al*., 1997), gave rise to high impact of food odour. Hence, apart from pyrazines, those in *Thua Nao* may contribute partially to its aroma.

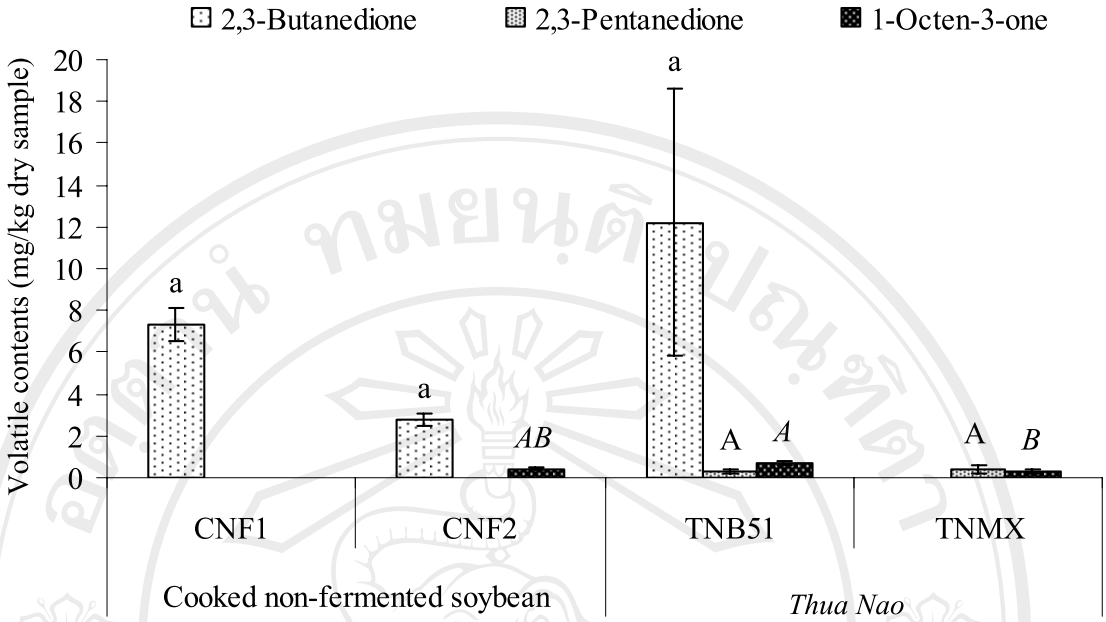

Figure 7.5 Ketones in cooked non-fermented soybeans and *Thua Nao*. CNF1, CNF2 = cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, *Thua Nao* was prepared by fermentation of boiled soybeans with naturally occurring microbes; TNB51, *Thua Nao* was prepared by fermentation of autoclaved soybeans with *B. subtilis* TN51. Each value represents mean ± SD (n = 3). Means (bar value) with different letters are significantly different ($P \leq 0.05$).

Sulphur-containing compounds have great influence on product odour even through they exist in such a very low concentration, since their threshold of olfactory detection is as low as 0.30 and 0.01 ppb for dimethyl disulfide and dimethyl trisulfide, respectively (Buttery *et al*., 1997). Usually these substances give rise to onion, sulphur and fishy odours (Lo *et al*., 2008). Table 7.1 and Figure 7.6 illustrate significantly higher sulphur-containing compounds in TNMX *Thua Nao* than those in TNB51 product ($P \leq 0.5$). However, these compounds in both *Thua Naos* presented exceeded the concentration of their threshold values. This result was also supported by Tanaka *et al*. (1998) who reported that home-made *Natto* also showed higher amount of sulphur-containing compounds than those in commercial *Natto* due to milder cooking temperature retaining different microbial strains.

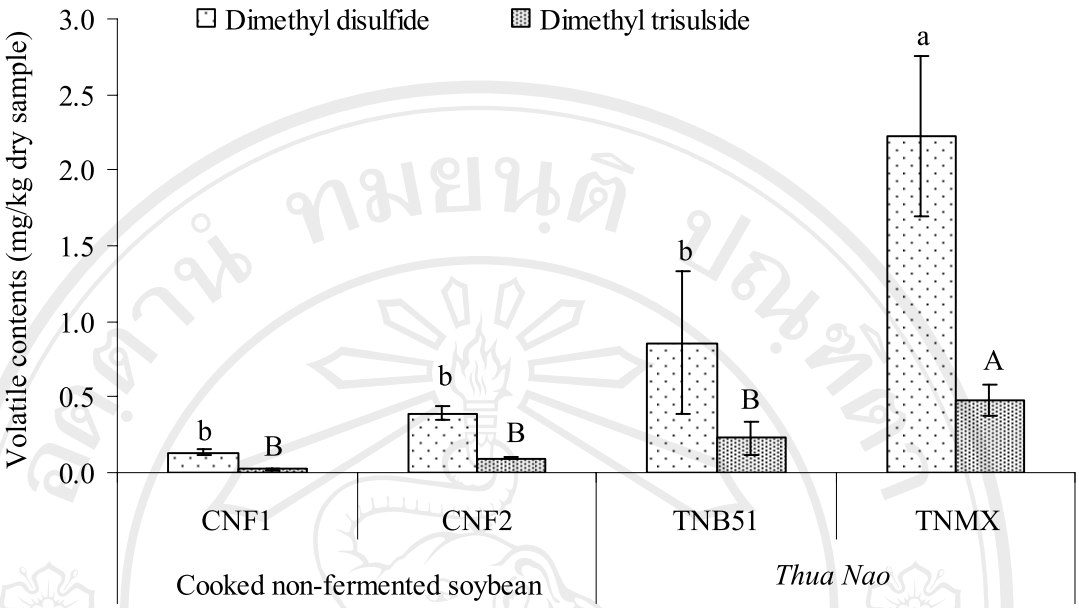

Figure 7.6 Sulphur-containing volatiles in cooked non-fermented soybeans and *Thua Nao*. CNF1, CNF2 = cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, *Thua Nao* was prepared by fermentation of boiled soybeans with naturally occurring microbes; TNB51, *Thua Nao* was prepared by fermentation of autoclaved soybeans with *B. subtilis* TN51. Each value represents mean ± SD (n = 3). Means (bar value) with different letters are significantly different ($P \leq 0.05$).

During fermentation, enzymatic activities produced by microbes such as protease, amylase, galactosidase and sucrase (Campbell-Platt, 1980; Odunfa, 1986) contribute the primary substances for product odour, as well as *Bacillus* sp. isolated from Thai traditional *Thua Nao* could produce several extracellular enzymes with the same function such as nattokinase, protease, amylase, phytase, lipases and glutamyl hydrolase (Chantawannakul *et al*., 2002; Visessanguan *et al*., 2005; Chukeatirote *et al*., 2006; Chunhachart *et al*., 2006; Dajanta *et al.,* 2009). These enzymatic degradation products such as dicarbonyl compounds and free amino acids will further generate complex odourous compounds through Strecker degradation and other reactions in Maillard browning. The predominant volatiles liberated from these interactions include pyrazines, aldehydes, alcohols, acids and esters. Precursors of

major volatile classes such as pyrazines derive from the interaction between amino acids and carbonyl compounds, for instance, threonine could be a precursor of 2,5-dimethylpyrazine, while tetramethylpyrazine was produced from different amino acids e.g. glycine, alanine, valine, isoleucine and leucine via Strecker degradation and deamination reaction (Shu, 1998). Tetramethylpyrazine was also derived from an interaction of acetoin (a by-product of the Maillard reaction) and ammonia (a by-product of amino acids hydrolysis) (Larroche *et al*., 1999).

7.4 Conclusion

In this study, the profile of volatile compounds responsible for the aroma of *Thua Nao* produced with pure and mixed cultures was determined using gas chromatography-mass spectrometry. In total, 85 volatile compounds were identified; these included pyrazines (10), aldyhydes (6), ketones (17), esters (8), alcohols (15), acids (4), aromatics (4), furans (5), sulphur-containing compounds (2), and other compounds (14). Generally, the predominant volatiles in fermented *Thua Nao* products were 2-methylbutanoic acid, 2,3,5-trimethylpyrazine, 2-methylpropanoic acid, acetic acid, 2,5-dimethylpyrazine, benzaldehyde, 5-methyl-3-hexanone, 2-butanone and 3-methyl-2-pentanone, whereas those in cooked non-fermented products (CNF) were ethanol, 2,3-butanedione, 3-hydroxy-2-butanone, acetone, benzaldehyde, and 2,5-dimethylpyrazine. It is therefore evident that there was a shift in volatile profiles from the CNF to fermented products. Besides, a different pattern of the volatile compounds was also observed in the *Thua Nao* samples prepared with pure and mixed starter culture.