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Study of volatile organic acids in freeze-dried *Cheonggukjang* formed during fermentation using SPME and stable-isotope dilution assay (SIDA)

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Abstract

Volatile organic acids in freeze-dried *Cheonggukjang* were quantified using a stable-isotope dilution assay (SIDA) according to the fermentation period. Five organic acids, acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid, and 3-methylbutanoic acid, were identified using solid-phase microextraction (SPME) in conjunction with gas chromatography–mass spectrometry (GC–MS). The contents of volatile organic acids in *Cheonggukjang* were highly dependent on the fermentation period and they increased during fermentation. Moreover, the branched-chained organic acids (namely 2-methylpropanoic acid and 3-methylbutanoic acid) were formed earlier and were present at much higher contents than the corresponding straight-chained organic acids during *Cheonggukjang* fermentation.

Keywords: *Cheonggukjang*; Stable-isotope dilution assay; Volatile organic acid; Gas chromatography–mass spectrometry (GC–MS); Solid-phase microextraction (SPME)

1. Introduction

Cheonggukjang is a traditional Korean food that has been a staple protein source for Koreans since ancient times. It provides, not only nutritional benefits, but also exhibits various physiological activities, such as antioxidation (Cheigh, Lee, & Lee, 1993), hypoglycemic and hypolipidemic effects (Kim, Kang, & Kwon, 2003), and fibrinolytic and immunostimulating activities (Chang, Shim, Kim, Chee, & Cha, 2005).

Cheonggukjang is normally produced from soybeans that are fermented for 2–3 days at 40–50 °C (Lee, 2001). It is similar to products produced by the fermentation of soybeans in other countries, such as *natto* (in Japan), *dawadawa* (in Africa), *thuanao* (in Thailand) and *kenima* (in India) (Lee, Koh, Yang, & Oh, 2001). The primary microorganism involved in the fermentation of *Cheonggukjang* is *Bacillus subtilis*, which produces the characteristic aromas, tastes and nutritional benefits.

Nowadays, the consumption of *Cheonggukjang*, which is sometimes distributed in dried forms for increased convenience and storage, is gradually increasing, mainly due to its various health benefits. However, even though *Cheonggukjang* has diverse health benefits, it is not so attractive to younger consumers, due to its unique strong off-odour note, which may be related to volatile components, such as butanoic acid, alkylpyrazines, ammonia, and sulfur-containing compounds produced by *Bacillus* species (Allaghency, Obanu, Campbell-Platt, & Owens, 1996; Youn et al., 2002). In particular, volatile organic acids, such as butanoic acid and 3-methylbutanoic acid, which have low threshold values and distinctive odours, sweaty and cheese-like, may be mainly responsible for the off-odour note of *Cheonggukjang*.

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Several studies have investigated the volatile compounds in *Cheonggukjang*, including those responsible for changes in flavour during fermentation (Choi & Ji, 1989, 1998), volatile compounds produced by different bacteria strains (Lee & Kim, 2004; Youn et al., 2002), and the effects of different fermentation methods and soybean cultivars (Choe, Yoo, Kim, & Chang, 1999). In particular, Choi and Ji (1989) investigated the changes of flavour in *Cheonggukjang* during fermentation, and they found that two volatile organic acids, 2-methylpropanoic acid and pentanoic acid, were important. Further volatile organic acids, such as acetic acid, propanoic acid, butanoic acid and 3-methylbutanoic acid, were subsequently identified by Choi et al. (1998). They explained that the quality of *Cheonggukjang* can be strongly affected by the contents of butanoic acid and 3-methylbutanoic acid.

The combined analytical technique of stable-isotope dilution assay (SIDA) and mass spectrometry (MS) is more accurate and sensitive than other quantitative methods (Rivero & Topiwala, 2004). Moreover, this method can compensate for any analytical errors occurring during sample preparation and instrumental analysis by using ^{2}D- or ^{13}C-labelled authentic standards, whose chemical and physical properties are nearly identical to those of unlabeled authentic standards. Several studies have reported that SIDA can be successfully applied to the quantification of volatile compounds in diverse foods, such as brewed coffee (Semmelroch & Grosch, 1996), strawberry juice (Schieberle & Hofmann, 1997), apple (Cunningham, Acree, Barnard, Butts, & Braell, 1986) and buttermilk (Heiler & Schieberle, 1997). It has also been shown that SIDA, combined with solid-phase microextraction (SPME), is a rapid and accurate quantitative method, especially, for low volatile and polar odorants, such as volatile organic acids (Blank, Milo, Lin, & Fay, 1999).

The aim of this investigation was to quantify the volatile organic acids in freeze-dried *Cheonggukjang*, using SPME combined with SIDA, according to the fermentation period.

2. Materials and methods

2.1. Materials

Cheonggukjang was prepared using a traditional method. Soybeans (Korean Bactae, *Glycine max* L.) were cultivated in Sunchang, Jeollanamdo, South Korea, in 2005. They were soaked in water at 15 °C for 15 h and steamed for 30 min at 118 °C. The steamed soybeans were cooled at 50 °C and then fermented at 42 °C for 46 h. The fermented samples were taken at various fermentation times, freeze-dried, and kept in a deep freezer (below −70 °C) before analysis.

Acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid and the internal standard compounds, [^{13}C]-acetic acid (99%), [$^{2}H_3$]-propanoic acid (99%), [$^{2}H_7$]-butanoic acid (98%) and 2-ethylbutanoic acid, were all purchased from Sigma–Aldrich. Inc. (St. Louis, MO, USA). An SPME fibre coated with 75 μm carboxen/polydimethylsiloxane (CAR/PDMS) was obtained from Supelco (Bellefonte, PA, USA).

2.2. Sample preparation

The samples (6.00 g) were ground using a pestle and mortar, and then transferred into a 60 ml vial. ^{2}D- or ^{13}C-labelled authentic compounds were dissolved in ethanol with concentrations adjusted to 60,000 ppm (w/v) for [^{13}C]-acetic acid, 5000 ppm (w/v) for [$^{2}H_3$]-propanoic acid, 500 ppm (w/v) for [$^{2}H_7$]-butanoic acid and 2000 ppm (w/v) for 2-ethylbutanoic acid. The mixture (500 μl) was added as an internal standard to the ground samples. They were then kept at 4 °C for 24 h before the extraction procedure.

2.3. Extraction of volatile organic acids

The volatile organic acids were extracted by SPME. The samples, stored in the 60 ml vials with a silicon/Teflon septum (Supelco, Bellefonte, PA, USA), were maintained at 75 °C for 1 h to obtain an equilibrium state. The volatile organic acids were absorbed for 30 min onto the CAR/PDMS-coated SPME fibre that was inserted (20 mm) into the sealed vial.

2.4. GC–MS analysis

GC–MS analysis was performed using an HP 5980A series GC-5972 mass selective detector (MSD) (Hewlett–Packard, Palo Alto, CA, USA) equipped with a DB-FFAP fused silica column (30 m length × 0.25 mm inner diameter × 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). The oven temperature was held at 50 °C for 8 min and then ramped to 160 °C (1 min) at a rate of 4 °C/min. The injector and detector temperatures were 250 and 280 °C, respectively. The monitored ions are listed in Table 1. The ionization energy was 70 eV.

Table 1
Analytical parameters used in stable-isotope dilution assay (SIDA)

Compounds	Selected ion (*m*/*z*)	Odour description[a]	Threshold value (μg/l)[b]	Internal standards	Selected ion (*m*/*z*)
Acetic acid	45	Sour, vinegar-like	200,000	[^{13}C]-Acetic acid	46
Propanoic acid	74	Sweaty	8100	[$^{2}H_3$]-Propanoic acid	77
2-Methylpropanoic acid	73	Sweaty	2300	[$^{2}H_3$]-Propanoic acid	77
Butanoic acid	60	Rancid cheese	173	[$^{2}H_7$]-Butanoic acid	63
3-Methylbutanoic acid	60	Sweaty	33.4	2-Ethylbutanoic acid	73

[a] Yonca et al. (2002).
[b] Water/ethanol solution (10%) containing 7 g/l of glycerine at pH 3.2 (Ferreira et al., 2000).

2.5. Quantitation of volatile organic acids

The amounts of five volatile organic acids, acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid and 3-methylbutanoic acid, in the samples were calculated from the peak area ratios for the unlabelled and labelled compounds versus the concentration ratio. For the study of acetic acid, which was unresolved with its corresponding internal standard, [^{13}C]-acetic acid, the abundances of 45 and 46 at m/z monitored for acetic acid and [^{13}C]-acetic acid were recalculated by subtracting the contamination from each other. The quantitative data are mean values of triplicate measurements. Table 1 shows the selective ions, odour descriptions, and threshold values of volatile organic acids in *Chenggukjang* and their internal standard compounds.

3. Results and discussion

Cheonggukjang is traditionally prepared by soaking, steaming, and fermenting soybeans in a humid closed space maintained at 40 °C for 2–3 days. During fermentation, proteins and carbohydrates are degraded by *B. subtilis*, which is the primary bacterium involved in *Cheonggukjang* fermentation, producing sugars, small peptides, and amino acids that contribute to the flavour and taste. In addition, sugars and amino acids are further metabolized during fermentation, forming pyruvic acid as a key intermediate in several organic acid-forming pathways (Skeie & Ardö, 2000). Organic acids are then produced by the Embden Meyerhof Parnas (EMP) pathway and hexose monophosphate pathway (HMP), in which pyruvic acid is a key intermediate. Pyruvic acid is a critical intermediate, leading to numerous volatile organic acids. It is also converted to lactic acid and several other organic acids during hetero-fermentation. Among the volatile organic acids found in *Cheonggukjang*, acetic acid, propanoic acid, and butanoic acid are formed from pyruvic acid *via* five or six successive reactions during the fermentation of propanoic acid and butanoic acid. Moreover, branched-chained organic acids, such as 2-methylpropanoic acid and 3-methylbutanoic acid, can be formed mainly from branched-chained amino acids, including valine, leucine and isoleucine. In particular, 2-methylpropanoic acid is produced from valine (Beck, 2005) and 3-methylbutanoic acid is formed from leucine catabolism *via* transamination, followed by oxidation steps (Czerny & Schieberle, 2005; Thierry, Richoux, & Kerjean, 2004).

Volatile organic acids, such as acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid and 3-methylbutanoic acid, in *Cheonggukjang* can make an important contribution to the characteristic flavour. In particular, butanoic acid and 3-methylbutanoic acid had relatively low threshold values, at 173 and 33.4 μg/l, respectively, in a water/ethanol solution (10%, v/v) containing 7 g/l of glycerine at pH 3.2 (Table 1). Also, they have characteristic odours, being rancid, sweaty, and cheese-like. Therefore, it is worth quantifying the contents of volatile organic acids that strongly affect the quality of *Cheonggukjang*. However, the quantitative analysis of volatile organic acids is not simple, mainly due to their high polarities. SIDA was used in this study to accurately quantify volatile organic acids. One of the main advantages of using SIDA is its high selectivity and the sensitivity obtained in the selected-ion monitoring (SIM) mode of GC–MS (Blank et al., 1999). SPME was employed to extract volatile analytes from samples. It requires a relatively simple sample preparation technique, using a fused silica fibre, which is coated on the outside with an appropriate stationary phase to absorb volatiles (Goupry, Rochut, Robins, & Gentil, 2000). The particular fibre used depends on the volatility and polarity of the analytes (Kataoka, Lord, & Pawliszyn, 2000). In general, a polar fibre is preferred for the extraction of polar analytes. Accordingly, a CAR/PDMS fibre has been successfully used to analyze volatile analytes with small molecular weights and high polarities (Roberts, Pollien, & Milo, 2000).

The quantitative data for volatile organic acids were obtained by comparing the abundances of each peak in GC–MS chromatograms in the SIM mode, as listed in Table 1. The contents of five volatile organic acids, namely acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid and 3-methylbutanoic acid, were determined in this study. The mass spectra of deuteriated butanoic acid and unlabelled butanoic acid are shown in Fig. 1. They indicate a shift, by three units, in the mass/charge ratio (m/z) in the fragment ($m/z = 63$) of labelled butanoic acid as compared to the corresponding fragment ($m/z = 60$) of the unlabelled butanoic acid. Accordingly, both deuteriated and unlabelled butanoic acid could be distinguished for quantitative analysis in GC–MS chromatograms. The contents of volatile organic acids in *Cheonggukjang* during fermentation are listed in Table 2. The contents of volatile organic acids were highly dependent on the fermentation period. In general, the contents of all volatile organic acids increased according to the fermentation period. After fermentation for 22 h, acetic acid, 2-methylpropanoic acid and 3-methylbutanoic acid increased significantly. This showed a growing tendency to produce volatile organic acids after 22 h. On the other hand, acetic acid was produced mainly at the beginning of fermentation (up to 7 h) and increased only gradually over the remainder of the fermentation period. It is notable that 2-methylpropanoic acid and propanoic acid were detected only after 22 h and 43 h of fermentation, respectively. Therefore, the branched volatile acids, 2-methylpropanoic acid and 3-methylbutanoic acid, were detected in the earlier part of the fermentation compared to their corresponding straight-chained acids. Branched-chained compounds have stronger and more characteristic odours than have straight-chained compounds and were present in higher amounts. Choi et al. (1998) also demonstrated that the contents of acetic acid, butanoic acid and 3-methylbutanoic acid were enhanced during fermentation. However, they reported

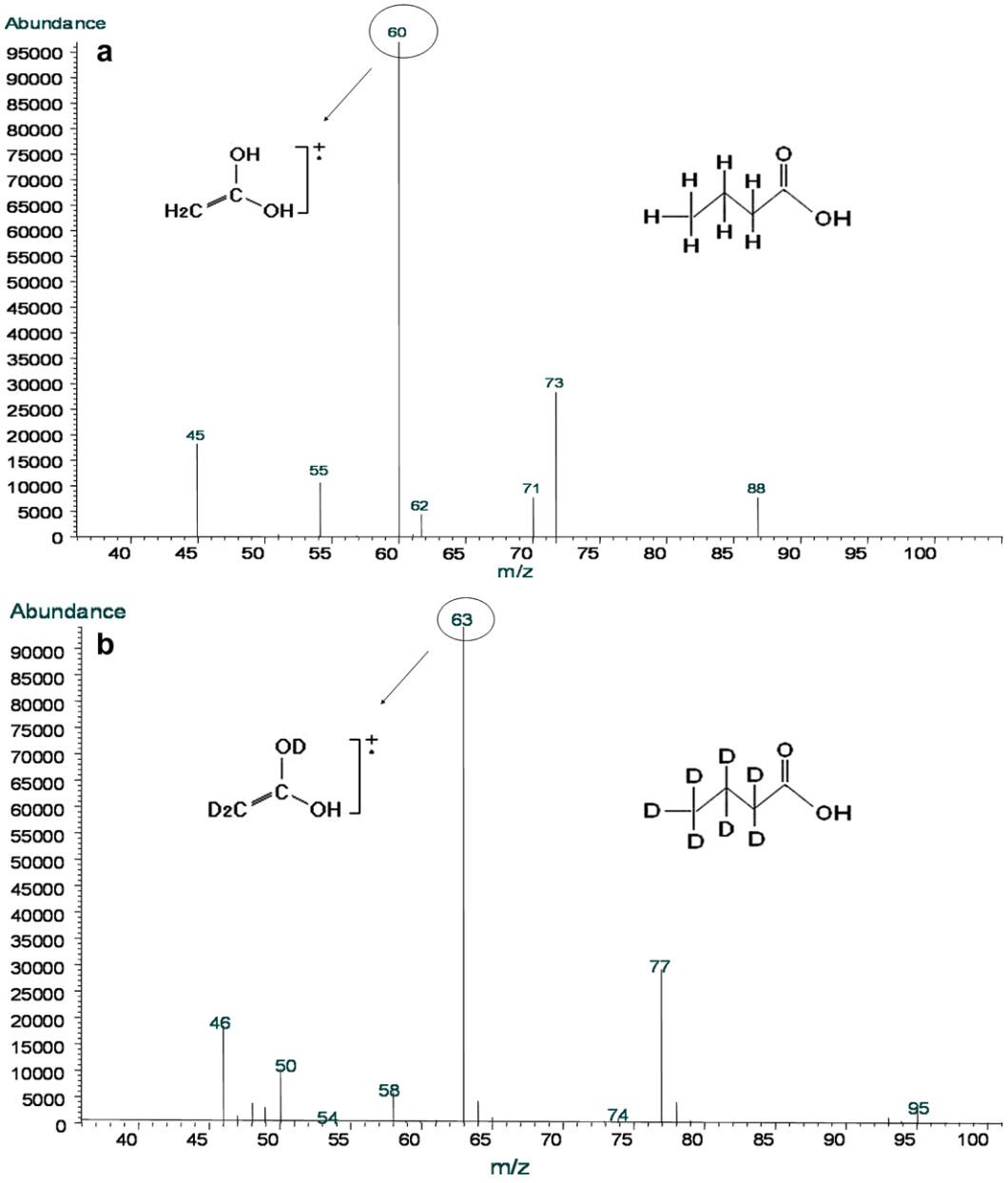

Fig. 1. Mass spectra of butanoic acid (a) and [$^2H_7$]-butanoic acid (b).

Table 2
The contents of volatile organic acids in *Cheonggukjang* during fermentation

Compounds	Contents (μg/100 g of dried sample)				
	0 h	7 h	22 h	31 h	43 h
Acetic acid	45.6 ± 1.510[a]	45.2 ± 0.544[a]	61.5 ± 1.713[b]	111 ± 1.733[c]	127 ± 1.693[d]
Propanoic acid	–	–	–	–	6.288 ± 0.065
2-Methyl propanoic acid	–	–	4.87 ± 0.195[a]	9.420 ± 0.790[b]	24.0 ± 2.294[c]
Butanoic acid	–	0.158 ± 0.003[a]	0.275 ± 0.017[a]	0.669 ± 0.051[b]	2.716 ± 0.182[c]
3-Methylbutanoic acid	0.293 ± 0.001[a]	0.306 ± 0.004[a]	1.15 ± 0.122[b]	4.32 ± 0.152[c]	15.30 ± 0.407[d]

Values with the same letter are not significantly different ($p < 0.05$).
[a] Means of three replicates ±SD.

that the content of propanoic acid decreased during fermentation; this discrepancy with the results of this study may be due to different cultivating conditions.

In conclusion, five volatile organic acids (acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid and 3-methylbutanoic acid) in *Cheonggukjang* were

quantified according to the fermentation period using SIDA combined with SPME. The formation of the volatile organic acids increased greatly during the fermentation period, and it was notable that the branched-chained organic acids, 2-methylpropanoic acid and 3-methylbutanoic acid, were detected earlier and were present at much higher concentrations than were the corresponding straight-chained organic acids.

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