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Changes of ethyl carbamate and its precursors in *maesil* (*Prunus mume*) extract during one-year fermentation

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ABSTRACT

The contents of ethyl carbamate (EC), cyanide, and ethanol were determined in *maesil* extracts that are liquids generated from fermentation of *maesil* fruit and brown sugar at 25 °C or 15 °C for one year. EC was detected from day 150 with a maximum value of 9.7 μg/kg. The cyanide levels increased with prolonged soaking time of *maesil* and decreased at day 150 where EC was firstly detected, indicating that cyanide is a precursor of EC. Ethanol slowly increased at 25 °C, while it fluctuated at 15 °C. The contents of EC, cyanide, and ethanol were higher in the extracts fermented at 25 °C compared with those at 15 °C. EC contents had a higher positive correlation with cyanide contents (R = 0.658) than ethanol contents (R = 0.351). These results indicate that fermenting temperature gave a rise of EC precursors and consequently led to the increase of EC in the *maesil* extract.

1. Introduction

Maesil is a Korean name for the fruit of *Prunus mume*, which is also called as Chinese plum or Japanese apricot. It has been used as a flavoring agent for alcoholic beverages, as a pickled food, and as a condiment in Korea, Japan, and China. Traditionally, *maesil* is not eaten raw owing to its poisonous cyanogenic glucoside amygdalin as well as sour taste (Bolarinwa, Orfila, & Morgan, 2014; Terada & Sakabe, 1988). Recently, the consumption of fermented *maesil* products has been rapidly increasing in Korea due to its claimed beneficial health effects, including its antioxidant and anti-osteoporosis activities (Yan et al., 2014).

Ethyl carbamate (EC) is naturally formed in fermented foods and alcoholic beverages during the fermentation process or during storage. It is well known that EC causes an increase in the incidence of tumors in several tissues, including lung, liver, and blood vessels (Beland et al., 2005; Mirvish, 1968; NTP, 1983). EC was recently upgraded to a probable human carcinogen (Group 2A) from a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC, 2007). The cyanogenic glycosides occurring mostly in the stone of *maesil* are enzymatically degraded to hydrocyanic acid, which is oxidized to cyanate. The cyanate reacts with ethanol generated through the fermentation of sugar by yeast and/or lactic acid bacteria, to form EC (Zimmerli & Schlatter, 1991). Choi and Koh (2015) reported that EC contents varied with the range from 3.4 μg/kg to 75.8 μg/kg in 31 *maesil* extracts, which consisted of 24 home-made samples and seven commercially available products in Korea.

The concern over the presence of EC and its toxicity in regularly consumed food products has raised interest in assessing the possible risks to human health. In addition to its precursors such as ethanol and cyanide, other factors including exposure to UV light, the storage time, and fermentation temperature have been known to affect the EC formation (Aresta, Boscolo, & Franco, 2001; Hansnip, Caputi, Crews, & Brereton, 2004; Riffikin, Wilson, Howie, & Muller, 1989). *Maesil* is commonly fermented with different manufacturing practices such as fruit soaking time and temperature. To the best of our knowledge, no data is available regarding changes of EC contents during *maesil* fermentation and factors influencing the EC formation in *maesil* extract.

Therefore, the objective of this study was to determine the levels of EC, cyanide, and ethanol in the liquid produced from the fermentation of *maesil* and brown sugar at 25 °C or 15 °C for one year in an effort to elucidate the effects of soaking time of *maesil*, temperature, and the ripening time on the EC formation.

2. Materials and methods

2.1. Chemicals and materials

Sodium hydroxide, sodium chloride, sodium bicarbonate, EC, acetone, potassium diphosphate, dipotassium phosphate, potas-

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sium cyanide, and β-glucosidase were purchased from Sigma-Aldrich (St Louis, MO, USA). The internal standard isotope labelled ethyl carbamate (EC-d_5) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Methylene chloride and picric acid were purchased from J. T. Baker (Center Valley, PA, USA) and BDH Ltd. (Poole, UK), respectively. Disposable diatomaceous Chem Elut column (50 mL) was purchased from Agilent Technology (Milwaukee, WI, USA).

2.2. Preparation of samples

Maesil fruits were purchased from a local *maesil* farm (Hadong, Korea) in May of 2014. The preparation procedure for *maesil* extract is presented in Fig. 1. The fruit was washed with distilled water, wiped with a cotton cloth, and then left at room temperature to remove the water remaining on the surface of fruit. The *maesil* (each 400 g) was mixed with brown sugar at a ratio of 1:1 (w/w) in a 1 L transparent plastic jar, in which the fruit was buried in sugar. Forty jars were placed into a thermostatic incubator (VS-1203PFC, Daejeon, Korea) set at 25 °C or 15 °C, which did not control the exposure to light in order to simulate a home-made fermentation. EC, ethanol, and cyanide levels were measured every 15 days for the first 90 days. At day 90, the fruits were taken out and the remaining liquid was ripened further for the next 275 days in the same jar. In comparison, another sample set continued to ferment with *maesil* in the jar. From day 90, EC, ethanol, and cyanide levels were measured every 30 days.

2.3. Analysis of EC

The EC level was determined according to AOAC method 994.07 with some modifications (AOAC, 2000). The EC-d_5 was used as an internal standard instead of n-propyl carbamate to improve a recovery correction. The gas chromatograph/mass spectrometer (GC/MS) conditions were revised to separate the EC-d_5 peak from the EC peak and shorten the run time. Stock solutions of EC and EC-d_5 were prepared at a concentration of 1000 μg/mL in acetone. Working solutions of EC were prepared at 7.5, 10, 20, 50, 100, 200, and 400 ng/mL by diluting the stock solution in acetone. The EC-d_5 at concentration of 400 ng/mL was prepared by the dilution of a stock solution in acetone. To spike it into the sample, the internal standard EC-d_5 was diluted with distilled water.

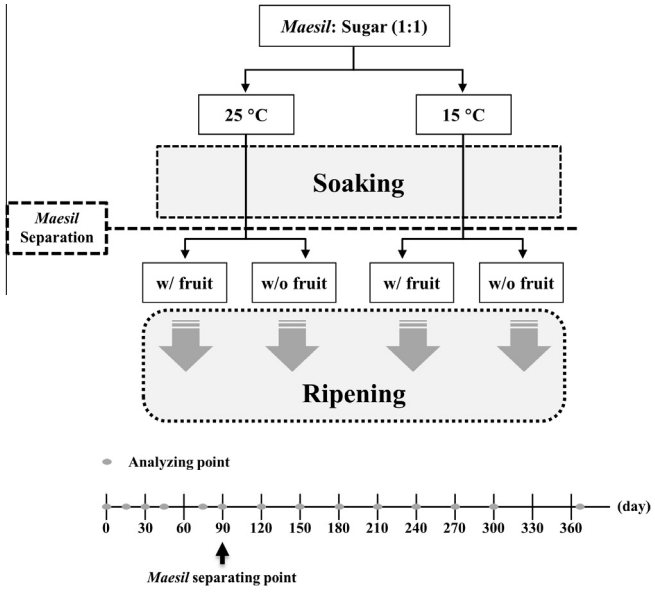

Fig. 1. Preparation procedure for *maesil* extract: w/ fruit symbolizes that it was fermented with *maesil* for 365 days. w/o fruit indicates that it was ripened after the *maesil* was taken out from the jar at day 90.

The *maesil* extract was initially neutralized with 1 N sodium hydroxide solution. 10 g of the neutralized sample was mixed with 30 mL of distilled water and 5 g of sodium chloride was then dissolved in it. After spiking 100 ng of EC-d_5, it was loaded into the Chem Elut column (Milwaukee, WI, USA). After 4 min of equilibration, EC was eluted with 160 mL of methylene chloride at a rate of 1 drop per second. The eluate was concentrated to about 2–3 mL using a rotary evaporator, transferred into a v-vial, and further concentrated to 1 mL using a dry block bath at 37 °C (EYELA MG-2200, Tokyo, Japan) under a gentle stream of nitrogen. Samples were analyzed in triplicate.

The concentrated sample was analyzed by means of GC/MS (Agilent Technologies Inc., Shanghai, China). A 7820A GC-5977E MS (Agilent Technologies, Santa Clara, CA, USA) instrument was used for the quantification and identification of EC. The GC conditions were as follows: capillary column 30 m length × 0.25 mm i.d., 0.25 μm film thickness DB-WAX (J&W, Folsom, CA, USA), helium carrier gas at 1 mL/min, injection volume of 1 μL in splitless mode, and an injector temperature of 210 °C. The oven temperature was as follows: 60 °C, 10 °C/min to 90 °C, 2 °C/min to 130 °C held for 5 min, 20 °C/min to 220 °C, followed by holding for 3 min. The MS was operated in the selected ion monitoring (SIM) mode with electron impact ionization (70 eV). The MS transfer line and ion source were kept at 240 °C and 230 °C, respectively. The mass to charge (m/z) levels of 62 and 64 were the major fragment ions of EC and EC-d_5. EC was quantified by using a standard curve obtained from the peak area ratios of m/z 62 and m/z 64 at seven standard concentrations. In addition, the peak was identified by comparing the area ratios of m/z 62 and m/z 74, which were the major fragment ions of EC.

2.4. Analysis of cyanide

Cyanide was determined using the enzyme-picrate acid method (Kim et al., 2010) and the methods of Egan, Yeah, and Bradbury (1998) and Bradbury, Egan, and Bradbury (1999). Standard stock solutions of cyanide were prepared at a concentration of 1000 μg/mL in 0.1 M phosphate buffer. Working solutions at concentrations of 0, 2, 10, 20, 50, and 100 μg/mL were prepared by diluting 500 μL of each stock solution in 0.1 M phosphate buffer. Picric paper was prepared by dipping a blotting paper (Whatman 3MM Chr, Kent, UK) into a picrate solution which consisted of 0.5% picric acid and 5% sodium bicarbonate in 40 mL of distilled water. The paper was dried in a dry oven for 10 min. The *maesil* fruit separated from the jars were freeze-dried at −50 °C at a pressure of 1.1 Pa for 48 h using a freeze-drier (EYELA FDU-1200, Tokyo Rikakikai, Tokyo, Japan). The dried *maesil* were ground using a Hibell Super Grinder (Hwaseong, Korea) and kept at −30 °C until extraction. The pulverized *maesil* (1 g) was dissolved in 19 mL of 0.1 M phosphate buffer. 2 mL of *maesil* solution was then mixed with 50 μL of β-glucosidase (3.43 unit) in a test tube. 500 μL of the *maesil* extract was treated as mentioned above. Picrate paper was placed into the tube and then kept for 3 h in a water bath (JSSB-330T, JS Research Inc., Gongju, Korea) at 45 °C. When the color of the paper changed to brown from yellow, the paper was taken from the tube and put in 3 mL of distilled water for 30 min. The absorbance of the solution was measured at 510 nm using a UV-Vis spectrophotometer (Biochrom Libra S22, Santa Barbara, CA, USA) against a blank of distilled water. Samples were analyzed in triplicate.

2.5. Analysis of ethanol

Ethanol was determined using an Alcolyzer Wine M/ME (Anton Parr GmbH, Graz, Austria) which patented the method based on