

Determination of D-saccharic acid-1,4-lactone (DSL) in fermentation tea (Kombucha) by capillary electrophoresis

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ABSTRACT

Kombucha or fermentation tea beverage produced from symbiosis of yeast species and acetic acid bacteria, is a popularly health tonic around the world today. D-saccharic acid-1,4-lactone (DSL), a component of kombucha, inhibits the activity of glucuronidase, known as an enzyme indirectly related with liver cancer. In this research, the tea fermentation was traditionally carried out by inoculating a previously grown culture into a freshly prepared tea decoction and incubated statically under aerobic conditions using 1.0% local tea from Northern Thailand, 10.0% sugarcane and 10.0% fungus broth at room temperature for 7-40 days, then filtered and pasteurization. After subsequent fermentation process procedure above, sampling was performed periodically in each jar. We founded various yeasts, acetic acid bacteria and lactic acid bacteria in floating cellulosic pellicle layer and the liquid broth after 7 days of fermentation. The ethanol content was determined by gas chromatography (GC) and Ebulliometer[®] was founded between 0.5-5.0% v/v. For safety of consumers, all samples were checked toxic substance such as methanol by using GC-MS. The total antioxidant of kombucha samples capacity was measured in terms of free radical-scavenging activity by the 1-diphenyl-2-picrylhydrazyl (DPPH) Radical decolorization method were between 0.221-0.512 mg gallic acid/ml and the total phenolic content was determined by the Folin-Ciocalteu method were between 0.291-0.854 mg gallic acid/ml. Using a capillary electrophoresis (CE) for the separation and determination of DSL in kombucha samples was carried out on CE System: PA 800 *plus* Pharmaceutical Analysis System (Beckman Coulter Inc., Brea, USA) with optimized conditions of 50 cm effective length capillary at a separating voltage of 30 kV in 40 mmol/l borax buffer (pH 6.5) containing 30 mmol/l SDS and 15% v/v methanol. The relationship between peak are and concentration of DSL was determined by UV absorption at wavelength 190 nm with the linear range of 25-200 µg/ml and a detection limit of 25 µg/ml. The electropherogram or fingerprint of the different fermented type's tea such as; green tea, oolong tea and black tea were established. We have successfully applied a simple CE method for quantitative evaluation of polyphenol or catechins and DSL in various fermented conditions and different kombucha products.

Keywords: D-saccharic acid-1,4-lactone, DSL, Fermentation tea, Kombucha, Capillary electrophoresis

1. INTRODUCTION

Kombucha, a fermentation tea beverage produced from a symbiosis of yeast species and acetic acid bacteria, has been favorite drink and becoming increasingly popular around the world today [1]. The beverage has been claimed to be beneficial to human health [2–3]. Some studies have detected the presence of polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins and antibiotics in the brewed tea [4–5].

β -Glucuronidase is an enzyme responsible for hydrolyzing glucuronides in the lumen of the gut. This reaction generates toxic and carcinogenic substances. β -Glucuronidase promotes cancerous formations by hydrolyzing conjugated glucuronides to carcinogenic aglyconic compounds and could be detoxified by glucuronide formation in the liver and then enter the bowel via bile. Thus, toxic aglycones are regenerated in situ in the bowel by bacterial β -Glucuronidase. d-Saccharic acid-1,4-lactone (DSL) is the competitive inhibitor of β -Glucuronidase [6]. DSL has been discovered in kombucha with varied concentrations and are considered to be the most healthful component in kombucha [7]. Effective identification and quantitative analysis of DSL in kombucha sample is of significance in explaining the beneficial effects of kombucha. In the past, several methods have been used in analyzing DSL and polyphenol components in brewed kombucha, such as; high-performance liquid chromatography (HPLC) [8], thin-layer chromatography (TLC) [9], mass spectrometry (MS) [10] and gas chromatography (GC) [11]. However, all these methods are required the complexities in sample pretreatment procedures. Up to today, there is only one report on the determination and separation of DSL in brewed kombucha samples using CE [12]. In this paper, a simple and selective CE method is established for the separation and quantitative determination of DSL in our brewed kombucha.

2. MATERIALS AND METHODS

Materials

d-Saccharic acid-1,4-lactone monohydrate ($C_6H_8O_7 \cdot H_2O$, analytical grade, >99.7%) (DSL), of which the molecular structure was shown in Figure 1, was purchased from Sigma (Germany). Methanol (HPLC grade, >99.9% purity) and Sodium dodecyl sulphate (SDS) were purchased from Merck, Darmstadt, Germany (analytical grade, >99.7%). Distilled, deionized water (Milli-Q Water Systems, Millipore Corporation, Bedford, MA, USA) was used to prepare all aqueous solutions. All other chemicals used were of analytical reagent grade. There were brewed kombucha samples from our laboratory (Lab of Microbiology, Faculty of Science, Chiang Mai University).

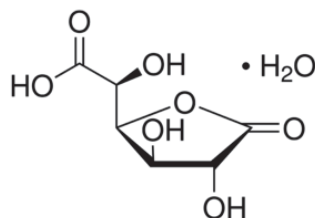


Figure 1. Chemical structure of d-saccharic acid-1,4-lactone monohydrate.

Instrumentation

All CE separations were conducted on a capillary electrophoresis system PA 800 *plus* Pharmaceutical Analysis System (Beckman Coulter Inc., Brea, USA) equipped with an autosampler and a DAD detector. The electrophoresis separation was performed on a fused-silica capillary of 75 μ m ID (375 μ m OD) \times 50 cm effective length.

Cultivation method

The kombucha tea was prepared using kombucha culture obtained from our laboratory. Tea samples were cultivated and received from Mae-sa-long highland, Chiang Rai, Thailand. Kombucha tea was prepared in a tea broth (1.0% w/v) supplemented with sucrose (10% w/v) by using our starter culture. Figure 2 showed the tea fungus in kombucha tea increased through 7 days of the fermentation. The subsequent fermentation process followed the procedure above up to 40 days. Sampling was performed periodically; each jar was sampled once only in order to avoid potential contamination. All analyses were carried out in duplicate.



Figure 2. Kombucha tea with tea fungus.

DSL Standard and Sample preparation

The DSL standard was prepared by quantitatively diluting the stock solution with pure water, and the stock solution was diluted to the desired concentrations just prior to use. Each kombucha sample was kept at -80°C . The samples were centrifuged at 10,000 rpm in 5 min. All samples were properly diluted, and duplicates were made.

Capillary electrophoresis

Running buffer was made up with 40 mM borax buffer ($\text{Na}_2\text{B}_4\text{O}_7\text{-KH}_2\text{PO}_4$), 30 mM SDS, 15% methanol (v/v), with pH of 6.52. Sample solutions, standard solutions and the running buffer were all filtered through a syringe cellulose acetate filter ($0.22\ \mu\text{m}$) prior to use. CE was performed at separation voltage of 30 kV with the running buffer. Sample injection was performed hydrodynamically for 10s. Capillary temperature was controlled at 25°C and detection wavelength was set at 190 nm. The capillary was washed with 0.1 mol/L NaOH and pure water for 2 min, respectively, and then rinsed with running buffer for 5 min before the next run.

3. RESULTS AND DISCUSSION

Microscopic evaluation revealed that the tea fungus mainly contains various yeasts, acetic acid bacteria and lactic acid bacteria in floating cellulosic pellicle layer and the liquid broth after 7 days of fermentation. During the fermentation process, yeasts and bacteria metabolize sucrose into a number of organic acids, such as acetic acid and gluconic acid. Due to an increased concentration of these organic acids, the pH decreased from 5 to 2.5. The ethanol content was determined by gas chromatography (GC) and Ebulliometer[®] was founded between 0.5-5.0 %v/v. For safety of consumers, all samples were checked toxic substance such as methanol by using GC-MS. The total antioxidant of kombucha samples capacity was measured in terms of free radical-scavenging activity by the 1-diphenyl-2-picrylhydrazyl (DPPH) Radical decolorization method were between 0.221-0.512 mg gallic acid/mL and the total phenolic content was determined by the Folin-Ciocalteu method were between 0.291-0.854 mg gallic acid/mL. It should be mentioned that from the antioxidant perspective, green tea kombucha are, generally more effective than black tea kombucha due to the higher amount of polyphenols or catechins.

According to the procedure of experiment, DSL separation and quantitative determination of DSL in kombucha samples were investigated. Standard curves were established from serial dilution of the stock DSL solutions. The relationship between peak area and the amount of substance applied, in a specified working range with linear response, was determined by first-order polynomial regression over the range 25–200 $\mu\text{g/mL}$ for DSL. The limit of detection (LOD) was at 25 $\mu\text{g/mL}$ with a signal-to-noise ratio of 3 ($S/N = 3$). The regression equation was $y = 0.002x + 10.62$, where y is the peak area and x the concentration of standard DSL ($\mu\text{g/mL}$) ($n = 3$, $R^2 = 0.998$). The method of addition of standard DSL sample to kombucha was used to prove qualitatively the effective separation of DSL and to determine the exact retention time of the eluted DSL peak. Electropherogram of kombucha tea after 7 days of fermentation was showed in Figure 3. Excellent baseline separation was obtained between DSL and other polyphenol components. Migration time for DSL was 14.6 min.

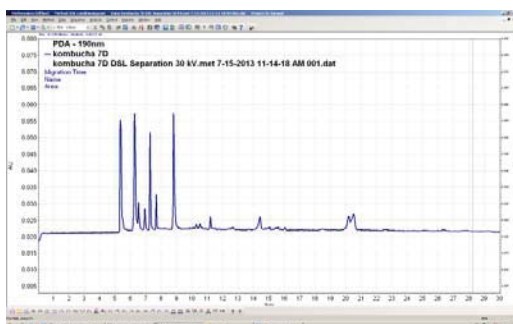


Figure 3. Electropherogram of kombucha tea after 7 days of fermentation.

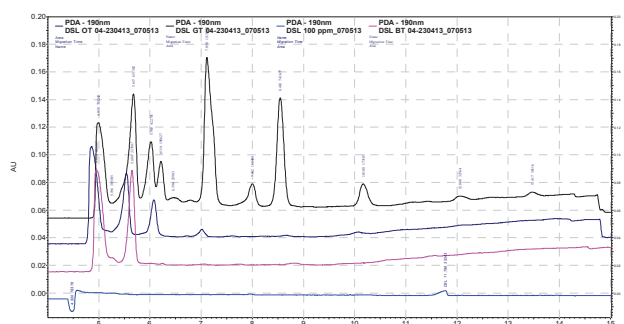


Figure 4. Electropherogram of green tea, oolong tea, black tea of kombucha samples and DSL standard.

In Figure 4, the electropherogram or fingerprint of the different fermented type's tea such as; green tea, oolong tea, black tea and DSL standard were established, from upper to lower, respectively. The separation performances under optimal conditions such as; pH, buffer, SDS concentration, and methanol concentration. In alkaline solutions (pH >8.0), the catechins or polyphenols were unstable and completely degraded within a few minutes [13]. The stability of catechins was increased in acidic solutions, but the speed of analysis was reduced for the slower electroosmotic flow. The pH value of running buffer was therefore chosen in neutral region, pH 6.52. Borax-phosphate was added into the running buffer to improve the separation-based on its complexation with the hydroxyl groups of tea polyphenols [14]. The borate concentration was selected to be 40 mM for optimum peak resolution, theoretical plates and shorter migration times. The use of a surfactant (SDS) might increase the resolution between DSL and other similar molecules in sample and therefore the migration time because of the negative charge of sulfate group of the SDS. Higher SDS concentration (40–50 mM) did not improve the peak resolution, while the electrophoretic current and migration times of the analytes were remarkably increased. For shorter migration times, better peak resolutions and fewer joule-heating problems, SDS concentration was selected to be 30mM. Organic modifiers were usually used to improve the separation [15]. On the other hand, an increase of concentration of organic solvent could enhance the solubility of DSL. Since organic solvents decrease the EOF and Joule's heat during electrophoresis, high voltage should be used to increase the resolution of electrophoresis. By adding methanol 15% (v/v), the resolution of polyphenols and DSL peaks were improved. With the optimized conditions, the kombucha samples can be analyzed within 15 min with a high theoretical plate number (>50,000) and a reduced electrophoretic current of about 30 μ A. More than 100 determinations were performed in the same capillary without significant changes in migration time, peak area and peak height. Compared with the running conditions of previous researches, the lower concentrations of SDS, phosphate and tetraborate buffer in this MEKC method were provided for a reduced the joule-heating problem and short the migration times of polyphenols and DSL [12,16-18]. Kombucha samples were determined by CE analysis; differences of DSL content between 25-75 μ g/mL were shown in different kombucha samples after incubation periods. This difference may be due to the different origins of microbial strains and different incubation conditions [19].

4. CONCLUSIONS

The results of this paper showed that CE method for the separation and determination of DSL in kombucha samples was highly selective, reproducible and simple to use. The main DSL and polyphenol constituents can be well separated in a 50 cm length capillary at a separation voltage of 30 kV in a 40 mmol/L borax buffer (pH 6.52) containing 30 mmol/L SDS and 15% methanol (v/v).

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